
2-D electrophoresis for proteomics
sample preparation discussion
the first dimension: isoelectric focusing (IEF)
the second dimension: SDS-PAGE
detection of proteins in gels
detection of proteins on western blots
image acquisition and analysis
identification and characterization of 2-D protein spots
sample solubilization and preparation methods
first-dimension separation methods
second-dimension separation methods
methods for protein detection in gels
troubleshooting guide
ordering information
references and related Bio-Rad literature
2-D Electrophoresis for Proteomics
A Methods and Product Manual
BIO-RAD
ProteomeWorksSystem.com

About This Manual

This manual is a guide to experimental approaches and methods in proteomics. As a reference tool, it provides a background on technologies and approaches that are general to all proteomics studies as well as sample protocols and tools to use as a starting point for most proteomics studies. It emphasizes how experimental conditions can be varied and interpreted to optimize your results, and also provides an extensive set of specialized references that you can consult for more information. Because each sample, experimental approach, and objective is different, this manual provides overall guidance on how to develop customized protocols suitable for the analysis of your samples.

The ProteomeWorks System

Two-dimensional (2-D) electrophoresis is an integral component of any proteomics program and is the core separation technology of the ProteomeWorks system. Because of its ability to separate and resolve complex mixtures of thousands of proteins in a single gel, 2-D electrophoresis has become the standard proteomics separation technique. Proteomics combines 2-D gel electrophoresis with high-throughput tools for image analysis, automated protein excision and digestion, and mass spectrometry identification. The ProteomeWorks system is a completely integrated system for protein discovery and proteomics analysis.

The ProteomeWorks system is the global alliance between Bio-Rad Laboratories, Inc. (USA) and Micromass, Ltd. (UK), dedicated to furthering proteomics research.

2-D Electrophoresis for Proteomics

A Methods and Products Manual

Two-Dimensional Electrophoresis for Proteomics Table of Contents

2-D electrophoresis for proteomics

sample preparation discussion

the first dimension: isoelectric focusing (IEF)

the second dimension: SDS-PAGE

detection of proteins in gels

detection of proteins on western blots

image acquisition and analysis

identification and characterization of 2-D protein spots

Part I
Discussion

Chapter 1 — Two-Dimensional Electrophoresis for Proteomics

The Context of Proteomics

Proteome analysis is a direct measurement of proteins in terms of their presence and relative abundance (Wilkins et al. 1996). The overall aim of a proteomic study is characterization of the complex network of cell regulation. Neither the genomic DNA code of an organism nor the amount of mRNA that is expressed for each gene product (protein) yields an accurate picture of the state of a living cell (Lubec et al. 1999), which can be altered by many conditions (Figure 1.1). Proteome analysis is required to determine which proteins have been conditionally expressed, how strongly, and whether any posttranslational modifications are affected. Two or more different states of a cell or an organism (e.g., healthy and diseased tissue) can be compared and an attempt made to identify specific qualitative and quantitative protein changes.

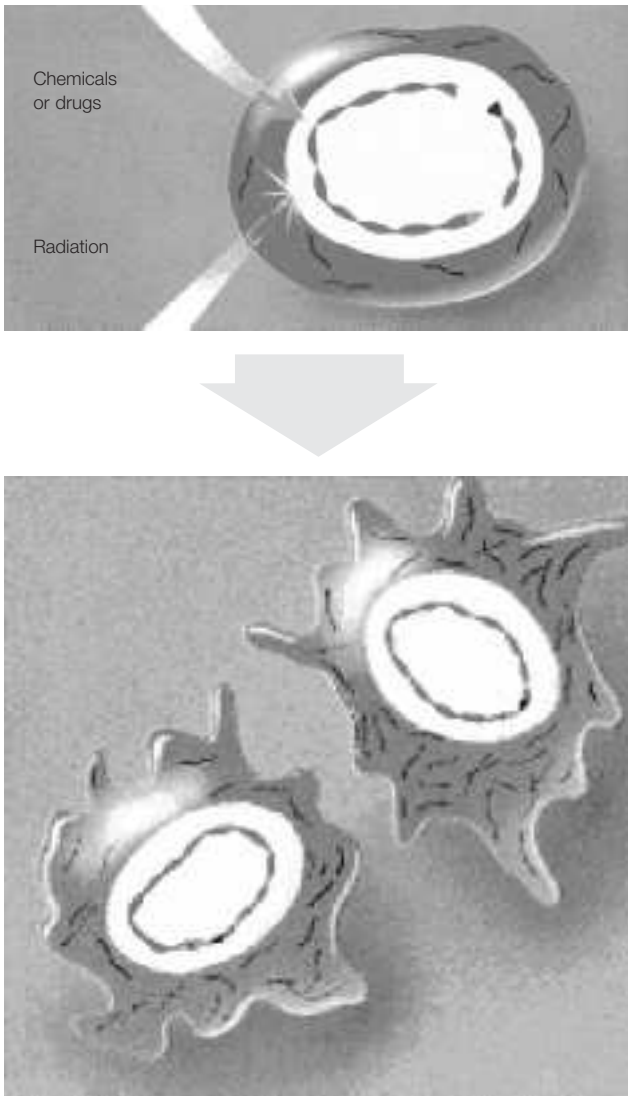

Fig. 1.1. Environmental or experimental perturbations can greatly change the proteins expressed in a cell, even when changes to the DNA code are minor or absent.

One of the greatest challenges of proteome analysis is the reproducible fractionation of these complex protein mixtures while retaining the qualitative and quantitative relationships. Currently, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is the only method that can handle this task (Cutler et al. 1999, Fegatella et al. 1999, Görg et al. 2000), and hence has gained special importance. Since 2-D PAGE is capable of resolving over 1,800 proteins in a single gel (Choe and Lee 2000), it is important as the primary tool of proteomics research where multiple proteins must be separated for parallel analysis. It allows hundreds to thousands of gene products to be analyzed simultaneously. In combination with computer-assisted image evaluation systems for comprehensive qualitative and quantitative examination of proteomes, this electrophoresis technique allows cataloging and comparison of data among groups of researchers.

Overview of Experimental Design

The general workflow (Figure 1.2) in a 2-D gel-based proteomics experiment and some of the factors affecting the way the experiment is performed are outlined below.

Sample Preparation

The method of sample preparation depends on the aim of the research and is key to the success of the experiment. Factors such as the solubility, size, charge, and isoelectric point (pI) of the proteins of interest enter into sample preparation. Sample preparation is also important in reducing the complexity of a protein mixture. The protein fraction to be loaded on a 2-D PAGE gel must be in a low ionic strength denaturing buffer that maintains the native charges of proteins and keeps them soluble. Chapter 2 (pages 3–7) discusses sample preparation.

First-Dimension Separation

Proteins are first separated on the basis of their pI, the pH at which a protein carries no net charge and will not migrate in an electrical field. The technique is called isoelectric focusing (IEF). For 2-D PAGE, IEF is best performed in an immobilized pH gradient (IPG). Chapter 3 (pages 8–12) discusses IEF.

Equilibration

A conditioning step is applied to proteins separated by IEF prior to the second-dimension run. This process reduces disulfide bonds and alkylates the resultant sulfhydryl groups of the cysteine residues. Concurrently, proteins are coated with SDS for separation on the basis of molecular weight (MW). Equilibration is discussed on page 14.

Second-Dimension Separation

The choice for the SDS-PAGE second-dimension gel depends on the protein MW range to be separated, as for 1-D PAGE. The ability to run many gels at the same time and under the same conditions is important for the purpose of gel-to-gel comparison. Discussion of second-dimension gels is found in Chapter 4 (pages 13–15).

Staining

In order to visualize proteins in gels, they must be stained in some manner. The exceptions are metabolically labeled proteins or iodinated proteins. The choice of staining method is determined by several factors, including desired sensitivity, linear range, ease of use, expense, and the type of imaging equipment available. At present there is no ideal universal stain. Sometimes proteins are detected after transfer to a membrane support by western blotting. These topics are discussed in Chapter 5 (pages 16–17) and Chapter 6 (pages 18–20).

Imaging

The ability to collect data in digital form is one of the major factors that enables 2-D gels to be a practical means of collecting proteome information. It allows unprejudiced comparison of gels, transfer of information among research groups, and cataloging of immense amounts of data. Many types of imaging devices interface with software designed specifically to collect, interpret, and compare proteomics data. Imaging equipment is briefly discussed in Chapter 7 (pages 21–22).

Image Analysis

Bio-Rad's PDQuest™ software and similar image analysis software packages compare gel images, annotate protein spots, and catalog data. PDQuest also drives Bio-Rad imaging instruments and the ProteomeWorks™ spot cutter. These software packages truly enable proteomic experiments by making comparison of large sets of data possible. Discussion of image analysis is found on page 22.

Protein Identification

Once interesting proteins are selected by differential analysis or other criteria, the proteins can be excised from gels and identified. The ability to precisely determine MW by mass spectrometry and to search databases for peptide mass matches have made high-throughput protein identification possible. One workflow procedure utilizes the ProteomeWorks family of products. The ProteomeWorks spot cutter automatically cuts resolved protein spots from gels with high precision and deposits them in the wells of microplates. The spot cutter can be operated independently or programmed to run from PDQuest software. The Mass*PREP* station robotically destains and digests excised proteins in preparation for mass spectrometry. Automatic peptide mass fingerprinting is done with Micromass' M@LDI spectrometer. Proteins not identified by MALDI can be identified by sequence tagging or de novo sequencing using the Q-Tof *Ultima* electrospray LC-MS-MS workstation. Alternative procedures can be found in several texts (Wilkins et al. 1997, Link 1999, Rabilloud 2000, Pennington and Dunn 2001).

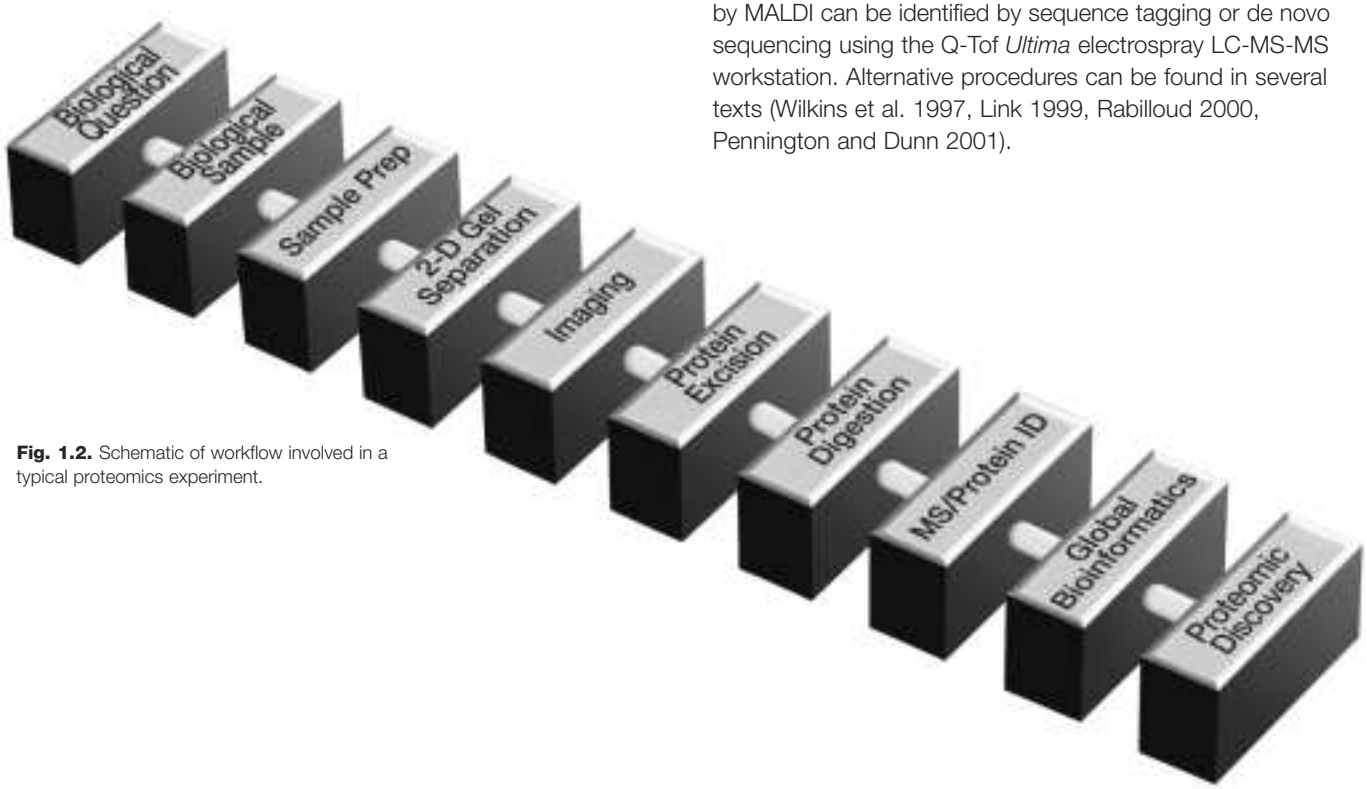

Fig. 1.2. Schematic of workflow involved in a typical proteomics experiment.

Chapter 2 — Sample Preparation Discussion

Efficient and reproducible sample preparation methods are key to successful 2-D electrophoresis (Link 1999, Rabilloud 1999, Macri et al. 2000, Molloy 2000). Sample preparation methods range from extraction with simple solubilization solutions to complex mixtures of chaotropic agents, detergents, and reducing agents. Sample preparation can include enrichment strategies for separating protein mixtures into reproducible fractions.

An effective sample preparation procedure will:

1. Reproducibly solubilize proteins of all classes, including hydrophobic proteins
2. Prevent protein aggregation and loss of solubility during focusing
3. Prevent postextraction chemical modification, including enzymatic or chemical degradation of the protein sample
4. Remove or thoroughly digest nucleic acids and other interfering molecules
5. Yield proteins of interest at detectable levels, which may require the removal of interfering abundant proteins or nonrelevant classes of proteins

Most protein mixtures will require some experimentation to determine optimum conditions for 2-D PAGE. Variations in the concentrations of chaotropic agents, detergents, ampholytes, and reducing agents can dramatically affect the 2-D pattern. Some examples follow.

Solubilization

Solubilization of proteins is achieved by the use of chaotropic agents, detergents, reducing agents, buffers, and ampholytes. These are chosen from a small list of compounds that meet the requirements, both chemically and electrically, for compatibility with the technique of IEF in IPG strips. The compounds chosen must not increase the ionic strength of the solution, to allow high voltages to be applied during focusing without producing high currents. The various components of sample buffers for IPG strips are discussed in the following paragraphs. See Chapter 9 for sample preparation procedures and solutions. See Table 2.1 for relevant products from Bio-Rad. Thorough discussion of solubilization methods, including new variations, can be found in several books (Link 1999, Pennington and Dunn 2000, Rabilloud 2000).

Chaotropic Agents

Urea is the most commonly used chaotropic agent in sample preparation for 2-D PAGE. Thiourea can be used to help solubilize many otherwise intractable proteins. Urea and thiourea disrupt hydrogen bonds and are used when hydrogen bonding causes unwanted aggregation or formation of secondary structures that affect protein mobility. Urea is typically used at 8 M. Thiourea is weakly soluble in water, but is more soluble in high concentrations of urea, so a mixture of 2 M thiourea and 5–8 M urea is used when strongly chaotropic conditions are required (Rabilloud 1998).

Table 2.1. Reagents for sample preparation by function.

Sample Preparation Products		Chaotropic Agent	Detergent	Reducing Agent	Buffer	Ampholyte
Individual Components						
161-0731	Urea, 1 kg	X	-	-	-	-
	Thiourea	X	-	-	-	-
161-0460	CHAPS, 1 g	-	X	-	-	-
161-0465	CHAPSO, 1 g	-	X	-	-	-
	SB 3-10	-	X	-	-	-
161-0407	Triton X-100, 500 ml	-	X	-	-	-
161-0611	Dithiothreitol, 5 g	-	-	X	-	-
163-2101	Tributylphosphine, 200 mM, 0.6 ml	-	-	X	-	-
161-0716	Tris, 500 g	-	-	-	X	
163-1112	Bio-Lyte® 3/10 Ampholyte, 40%, 10 ml	-	-	-	-	X
163-1132	Bio-Lyte 3/5 Ampholyte, 20%, 10 ml	-	-	-	-	X
163-1142	Bio-Lyte 4/6 Ampholyte, 40%, 10 ml	-	-	-	-	X
163-1152	Bio-Lyte 5/7 Ampholyte, 40%, 10 ml	-	-	-	-	X
163-1192	Bio-Lyte 5/8 Ampholyte, 40%, 10 ml	-	-	-	-	X
163-1162	Bio-Lyte 6/8 Ampholyte, 40%, 10 ml	-	-	-	-	X
163-1172	Bio-Lyte 7/9 Ampholyte, 40%, 10 ml	-	-	-	-	X
163-1182	Bio-Lyte 8/10 Ampholyte, 20%, 10 ml	-	-	-	-	X
Bio-Lyte IEF Buffers*						
163-2093	100x ReadyStrip 7–10 Buffer, 1 ml	-	-	-	-	X
163-2094	100x Bio-Lyte 3/10 Ampholyte, 1 ml	-	-	-	-	X
163-2095	100x ReadyStrip 6.3–8.3 Buffer, 1 ml	-	-	-	-	X
163-2096	100x ReadyStrip™ 5.5–6.7 Buffer, 1 ml	-	-	-	-	X
163-2097	100x ReadyStrip 4.7–5.9 Buffer, 1 ml	-	-	-	-	X
163-2098	100x ReadyStrip 3.9–5.1 Buffer, 1 ml	-	-	-	-	X
Solutions or Kits						
163-2100	ReadyPrep™ Sequential Extraction Kit	X	X	X	X	X
163-2102	ReadyPrep Reagent 1, 1 vial	-	-	-	X	-
163-2103	ReadyPrep Reagent 2, 1 vial	X	X	X	X	X
163-2104	ReadyPrep Reagent 3, 1 vial	X	X	X	X	X
163-2105	ReadyPrep 2-D Starter Kit	X	X	X	X	X
163-2106	ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer	X	X	X	X	X

* Dilute ReadyStrip buffers to 1x final in each sample to equal 0.2% Bio-Lyte ampholyte.

For methods related to this section see page 27

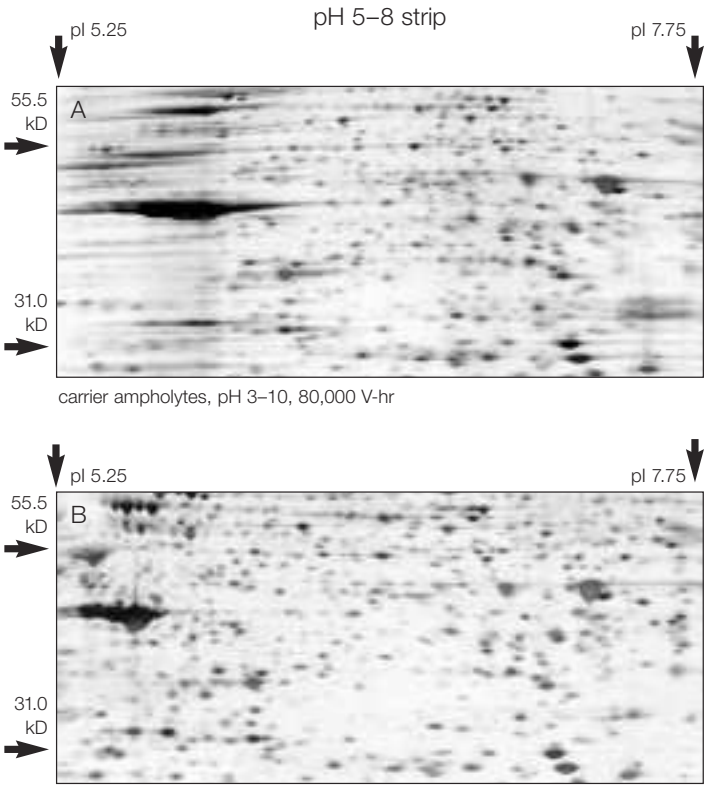

Fig. 2.1. Effect of ampholytes on resolution. Matching sections of 2-D images are shown. In both A and B, 110 µg of a cytosolic extract of a human lymphoblastoid cell line was passively loaded into a 17 cm pH 5–8 ReadyStrip IPG strip. Second-dimension separation was in 10–24% gradient gels with PDA crosslinker in PROTEAN® II XL format. In A, pH 3–10 carrier ampholytes were used. In B, pH 5–8 carrier ampholytes were mixed with pH 8–10 carrier ampholytes at a 2:1 ratio. The use of the ampholyte mixture greatly improved focusing. Data kindly provided by R Joubert-Caron, Laboratoire de Biochimie des Proteines et Proteomique.

Detergents

Detergents are added to disrupt hydrophobic interactions and increase solubility of proteins at their pI. Detergents must be nonionic or zwitterionic to allow proteins to migrate according to their own charges. Some proteins, especially membrane proteins, require detergents for solubilization during isolation and to maintain solubility during focusing. Ionic detergents such as SDS are not compatible with IEF, but can be used with concentrated samples in situations where the SDS can be unbound from the proteins by IEF-compatible detergents that compete for binding sites. Nonionic detergents such as octylglucoside, and zwitterionic detergents such as CHAPS and its hydroxyl analog, CHAPSO, can be used. CHAPS, CHAPSO, or octylglucoside concentrations of 1–2% are recommended (Rabilloud 1999). New detergents are emerging that have great potential in proteomics, including SB 3-10 and ASB-14 (Chevallet et al. 1998). Some proteins may require detergent concentrations as high as 4% for solubility (Hermann et al. 2000).

Carrier Ampholytes

A fundamental challenge with IEF is that some proteins tend to precipitate at their pI. Even in the presence of detergents, certain samples may have stringent salt requirements to maintain the solubility of some proteins. Salt should be present in a sample only if it is an absolute requirement, and then only at a total concentration less than 40 mM. This is problematic since any salt included will be removed during the initial high-current stage of focusing. Salt limits the voltage that can be achieved without producing high current, increasing the time required for focusing. Proteins that require salt for solubility are subject to precipitation once the salt is removed. Carrier ampholytes sometimes help to counteract insufficient salt in a sample. They are usually included at a concentration of ≤0.2% (w/v) in sample solutions for IPG strips. High concentrations of carrier ampholytes will slow down IEF until they are focused at their pI, since they carry current and hence limit voltage.

Some researchers have increased resolution by varying the ampholyte composition. An example is shown in Figure 2.1, where the resolution in the first dimension is greatly increased by using a mixture of ampholytes. See Table 2.1 for relevant products from Bio-Rad.

For ordering information related to this section see page 45

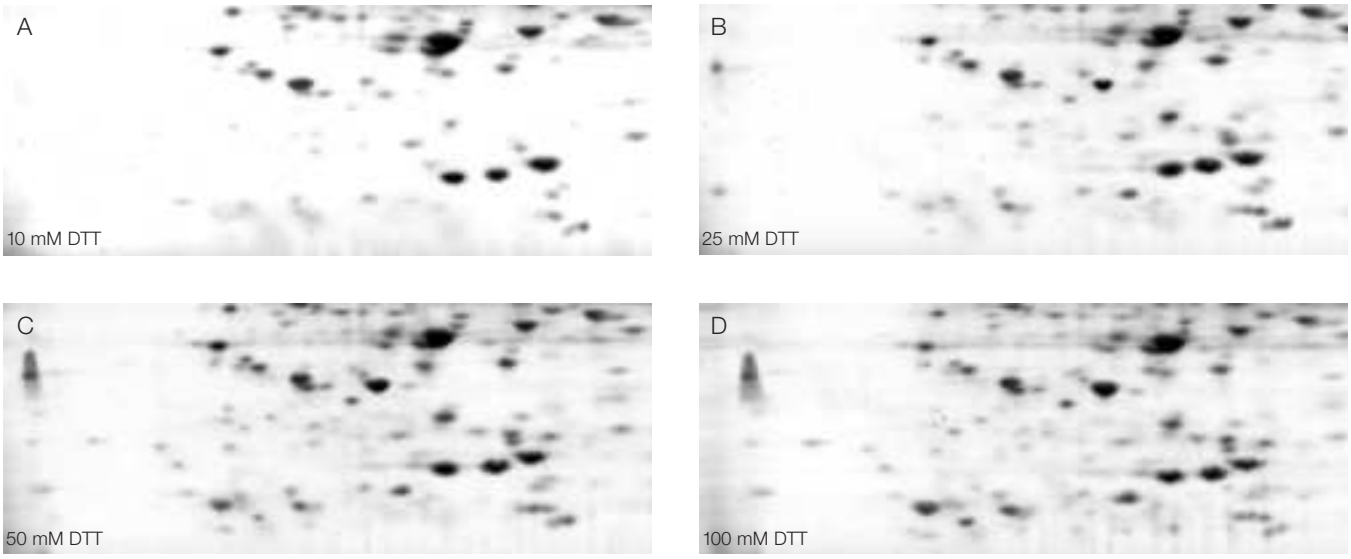

Fig. 2.2. Effect of DTT concentration on 2-D protein spot pattern. A, 200 µg of *E. coli* extract was suspended in rehydration buffer containing 10 mM DTT and subjected to 2-D gel electrophoresis (first dimension in 11 cm, pH 4–7 IPG for 40,000 V-hr, second dimension in 8–16% SDS-PAGE gel). In B, C, and D, rehydration buffer included 25 mM, 50 mM, and 100 mM DTT, respectively. The gels were stained with Bio-Safe™ Coomassie Blue stain. The images show the acidic, low MW regions of each gel. Notice that as the DTT concentration was increased, the number of spots resolved in this region also increased, indicating that 10 mM DTT is insufficient to completely reduce the disulfides present in the protein mixture. Data kindly provided by William Strong of Bio-Rad Laboratories.

Reducing Agents

Reducing agents such as dithiothreitol (DTT) or tributyl-phosphine (TBP) are used to disrupt disulfide bonds. Bond disruption is important for analyzing proteins as single subunits. DTT is a thiol reducing agent added in excess to force equilibrium toward reduced cysteines. At 50 mM it is effective in reducing most cystines, but some proteins are not completely reduced by this treatment. If the concentration of DTT is too high it can affect the pH gradient since its pK_a is around 8. Figure 2.2 shows the effect of DTT concentration on samples of soluble *E. coli* proteins. The result will be different for samples from different sources.

TBP is a much more effective reducing agent than DTT. It reacts to reduce cystines stoichiometrically at low millimolar concentrations (Herbert et al. 1998). It is chemically more difficult to handle than DTT, but Bio-Rad has solved this problem by supplying it in a form safe for shipping and lab use. See Table 2.1 for these reducing agents from Bio-Rad.

For a more thorough discussion of the effects of detergents, denaturing agents, and reducing agents on protein solubility, consult the following papers: Rabilloud (1998, 1999), Herbert et al. (1998), Molloy (2000), and Taylor et al. (2000).

Prefractionation

Reducing the complexity of the sample loaded on a 2-D gel can increase the visibility of minor proteins. Techniques such as differential extraction (Molloy et al. 1998), subcellular fractionation (Taylor et al. 2000, Morel et al. 2000), chromatography (Fountoulakis et al. 1999), or prefocusing in a preparative IEF device such as the Rotofor® system (Masuoka et al. 1998, Nilsson et al. 2000) have been used to reduce the complexity of samples.

Removal of Albumin and IgG

The isolation of lower-abundance proteins from serum or plasma is often complicated by the presence of albumin and immunoglobulin G (IgG). Albumin is the most abundant protein (~60–70%) in serum and IgG is the second most abundant protein (10–20%). These two proteins effectively act as major contaminants, masking the presence of many comigrating proteins as well as limiting the amount of total serum protein that can be resolved on a 2-D gel.

In the past, removal of albumin and IgG usually required separate chromatography methodologies for each of the two species. Now, Bio-Rad's Aurum™ serum protein kit allows selective binding and simultaneous removal of both albumin and IgG from serum or plasma samples prior to 2-D electrophoresis. By using a single column for purification,

For methods related to this section see page 28

the process retains precious samples for subsequent gel analysis. The removal of these major proteins reduces the total serum protein by 70% or more, so that 3–4 times more of the enriched serum sample can be loaded. Low-abundance proteins can thus be visualized; in addition, proteins that comigrate with albumin and IgG are better resolved. Figure 2.3 shows the albumin and IgG removal capability of the Aurum serum protein kit.

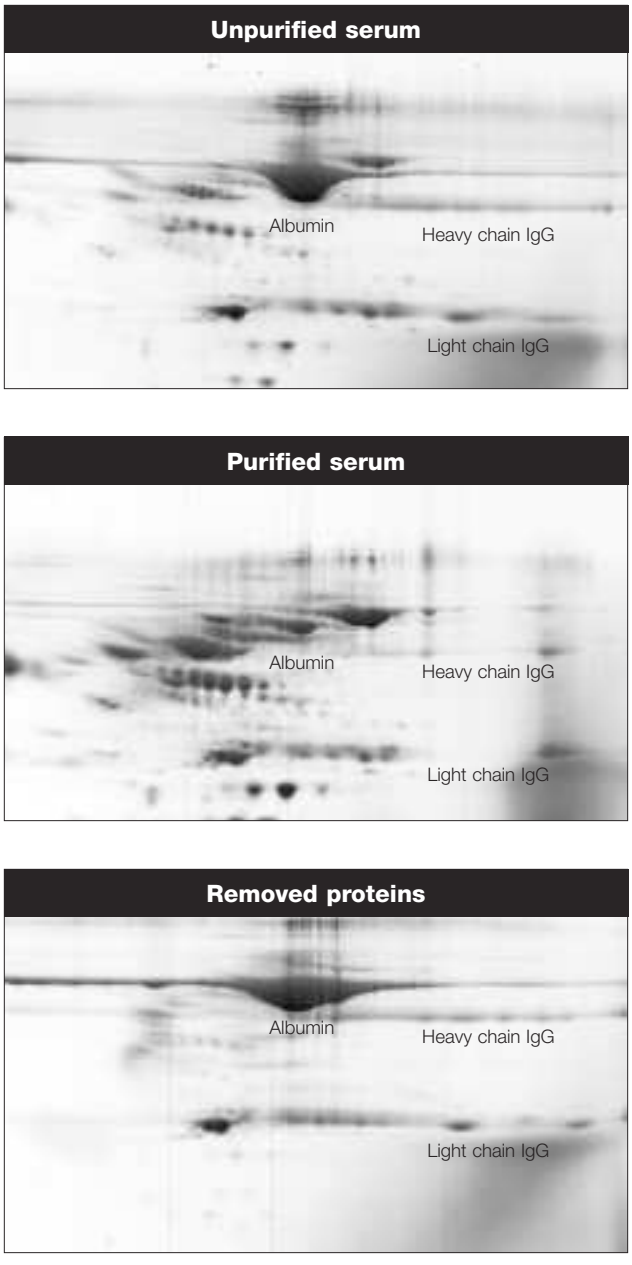

Fig. 2.3. Total protein (1.32 mg) was loaded onto an Aurum serum protein mini column. Then 200 µg total protein from each fraction (unpurified serum, purified serum, removed proteins) was loaded onto ReadyStrip IPG strips (pH 3–10, 11 cm). Criterion™ precast gels (8–16%, 4% stacking gel, Tris-HCl, 13.3 x 8.7 cm) were run at 200 V for 1 hr in a Criterion Dodeca™ cell and stained with Bio-Safe™ Coomassie stain. Images were acquired on the GS-800 calibrated densitometer using PDQuest™ 2-D analysis software.

Sequential Extraction

One method for reducing sample complexity is the basis of the ReadyPrep™ sequential extraction kit. This protocol takes advantage of solubility as a third independent means of protein separation. Proteins are sequentially extracted in increasingly powerful solubilizing solutions. More protein spots are resolved by applying each solubility class to a separate gel, thereby enriching for particular proteins while simplifying the 2-D patterns in each gel. An increase in the total number of proteins is detected using this approach (Molloy et al. 1998).

The reagents may be prepared by the protocols provided in Chapter 9, or purchased from Bio-Rad as the ReadyPrep sequential extraction kit (see Table 2.1). Each of the three reagents solubilizes an overlapping set of proteins, as illustrated by the flowchart in Figure 2.4 and by the results in Figure 2.5. Reagent 1 extracts soluble proteins, such as cytosolic proteins. Reagent 2 is used to extract proteins of intermediate solubility, while reagent 3 extracts proteins insoluble in reagents 1 and 2. See page 28 for the protocol.

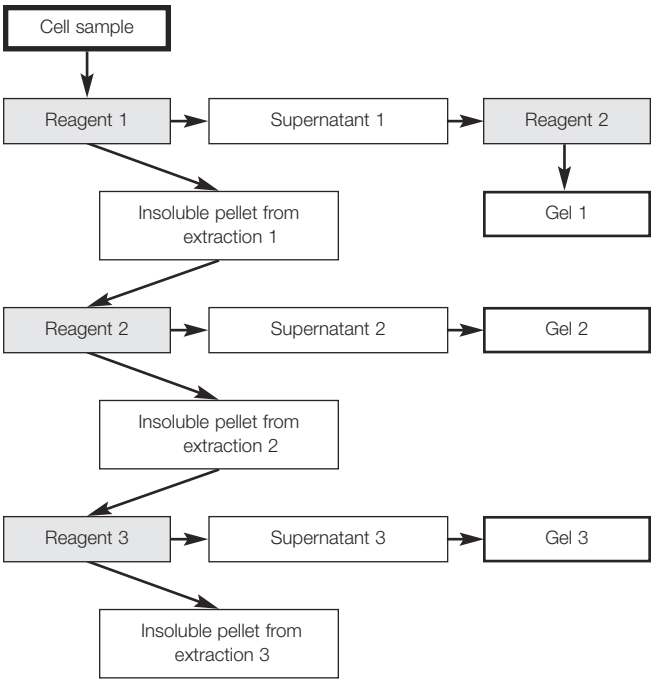

Fig. 2.4. Flowchart for sequential extraction of proteins.

For ordering information related to this section see page 45

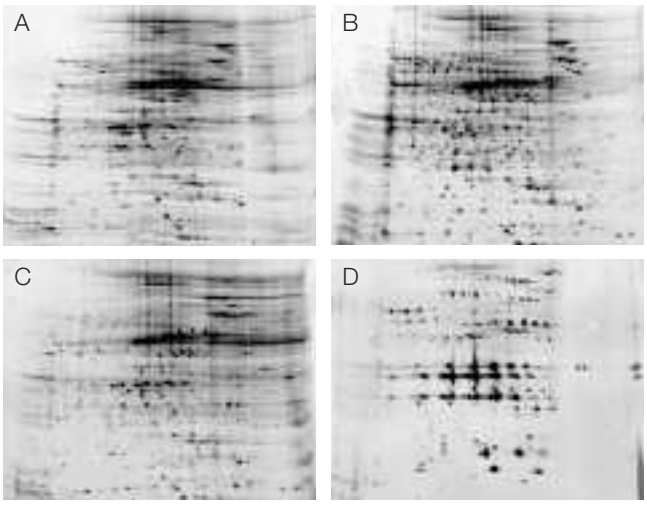

Fig. 2.5. Increased protein display in sequentially extracted *E. coli* cell pellet. *E. coli* strain W3110 was collected by centrifugation. The cell pellet was suspended in reagent 1 and the cells were lysed by sonication. One portion of the sonicated cell suspension, containing 200 µg of protein, was diluted in ReadyPrep extraction reagent 3. The proteins soluble in reagent 3 were termed a whole-cell extract. Another portion of the sonicated cell suspension was sequentially extracted. First-dimension separation was by IEF from pH 4–7 in an IPG gel. The second-dimension separation was by SDS-PAGE in an 8–16%T polyacrylamide gradient gel. A, 2-D PAGE of whole-cell extract; B, 2-D PAGE of 200 µg of protein solubilized with reagent 1; C, 2-D PAGE of 200 µg of protein solubilized with reagent 2; D, 2-D PAGE of 200 µg of protein solubilized with reagent 3.

Removal of DNA

The presence of nucleic acids, especially DNA, interferes with separation of proteins by IEF. Under denaturing conditions, DNA complexes are dissociated and markedly increase the viscosity of the solution, which inhibits protein entry and slows migration in the IPG. In addition, DNA binds to proteins in the sample and causes artifactual migration and streaking.

The simplest method for removal of DNA is enzymatic digestion. Adding endonuclease to the sample after solubilization at high pH (40 mM Tris) allows efficient digestion of nucleic acids while minimizing the action of contaminating proteases. The advantage of the endonuclease method is that sample preparation can be achieved in a single step, by the addition of the enzyme prior to loading the first-dimension IPG. See page 29 for the experimental protocol.

Protein Load

The amount of protein applied to an IPG strip (see page 8) can range from several micrograms to 1 mg or more (Bjellqvist et al. 1993b; see also page 32). Some of the factors affecting the decision of how much protein to load are:

- Subsequent analysis. Enough of the protein of interest must be loaded for it to be analyzed. With the Ready Gel® mini system (7 cm IPG), detection of moderately abundant proteins in complex mixtures with Coomassie Brilliant Blue R-250 dye requires on the order of 100 µg total protein. With the same load, many low-abundance proteins can be detected with more sensitive stains such as silver or SYPRO Ruby protein gel stain. See Table 5.1 on page 16 for sensitivity of stains.
- The purpose of the gel. If the gel is being run solely for the sake of getting a good image of well-resolved proteins for comparative studies or for publication, the protein load would be the minimum amount that is stainable.
- The abundance of the proteins of interest. If the purpose is to study low-copy-number proteins, a large mass of a protein mixture might be loaded (Wilkins et al. 1998).
- The complexity of the sample. A highly complex sample containing many proteins of widely varying concentrations might requre a compromise load so that high-abundance proteins don't obscure low-abundance proteins.

  By enriching a sample for specific types of proteins using prefractionation techniques, each individual protein will be at a higher relative concentration, which means that enough material can be loaded for detection of low-abundance constitutents. Examples are: a fraction obtained by differential solubility, a chromatography fraction, a Rotofor fraction, or any subcellular organelle fraction.
- pH range of IPG strip. In general, larger amounts of total protein can be loaded on a narrow-range IPG strip. Only the proteins with a pI within the strip pH range will be represented within the second-dimension gel.

Preventing Keratin Contamination

Careful sample handling is important when sensitive detection methods are employed. Silver-stained SDS-PAGE gels sometimes show artifacts in the 50 to 70 kD region and irregular but distinctive vertical streaking parallel to the direction of migration. This has been attributed to the reduction of skin keratin, a contaminant inadvertently introduced into the samples (keratin in the sample solution usually is focused near pH 5). Skin keratin is also a common contaminant seen in mass spectra. The best remedy for the keratin artifact is to avoid introducing it into the sample in the first place. Monomer solutions, stock sample buffers, gel buffers, and electrode buffers should be filtered through nitrocellulose and stored in well-cleaned containers. It also helps to clean the gel apparatus thoroughly with detergent and to wear gloves while assembling the equipment.

Additional Resources

Samples can be prepared for 2-D electrophoresis using many other techniques. The scope of such a discussion is much too broad for this booklet. The resources cited on the following topics should be consulted for further information:

- Cell disruption (Deutscher 1990, Bollag et al. 1996, Link 1999)
- Immunoprecipitation (Harlow and Lane 1988)
- Plant cell sample preparation (Link 1999)
- Subcellular organelles (Celis 1998)
- Microdissection (Celis et al. 1999)

For methods related to this section see page 32

Chapter 3 — The First Dimension: Isoelectric Focusing (IEF)

Isoelectric Point (pI)

Differences in proteins' pI are the basis of separation by IEF. The pI is defined as the pH at which a protein will not migrate in an electric field and is determined by the number and types of charged groups in a protein. Proteins are amphoteric molecules. As such, they can carry positive, negative, or zero net charge depending on the pH of their local environment. For every protein there is a specific pH at which its net charge is zero; this is its pI. Proteins show considerable variation in pI, although pI values usually fall in the range of pH 3–12, with the majority falling between pH 4 and pH 7. A protein is positively charged in solution at pH values below its pI and negatively charged at pH values above its pI.

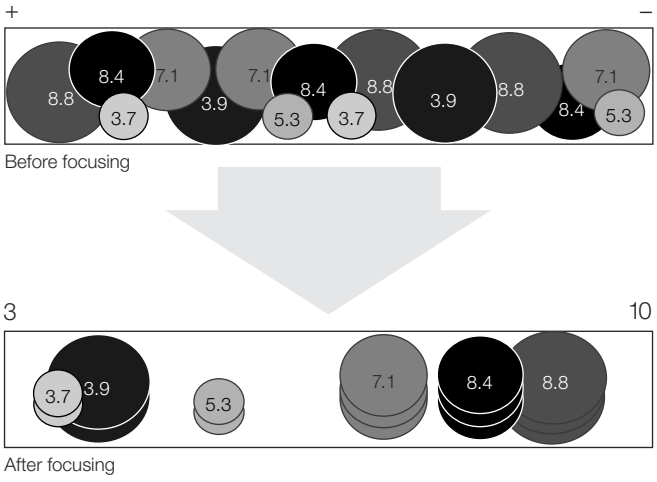

Fig. 3.1. A mixture of proteins is resolved on a pH 3–10 IPG strip according to each protein's pI and independently of its size, as described in the IEF section.

IEF

When a protein is placed in a medium with a pH gradient and subjected to an electric field, it will initially move toward the electrode with the opposite charge. During migration through the pH gradient, the protein will either pick up or lose protons. As it migrates, its net charge and mobility will decrease and the protein will slow down. Eventually, the protein will arrive at the point in the pH gradient equal to its pI. There, being uncharged, it will stop migrating (Figure 3.1). If this protein should happen to diffuse to a region of lower pH, it will become protonated and be forced back toward the cathode by the electric field. If, on the other hand, it diffuses into a region of pH greater than its pI, the protein will become negatively charged and will be driven toward the anode. In this way, proteins condense, or are focused, into sharp bands in the pH gradient at their individual characteristic pI values.

Focusing is a steady-state mechanism with regard to pH. Proteins approach their respective pI values at differing rates but remain relatively fixed at those pH values for extended periods. By contrast, proteins in conventional electrophoresis continue to move through the medium until the electric field is removed. Moreover, in IEF, proteins migrate to their steady-state positions from anywhere in the system.

IPGs Versus Carrier Ampholytes

IEF, either using IPG strips or using carrier ampholytes and tube gels, may be used to resolve proteins in the first dimension (Garfin 2000). The IPG method has numerous advantages (summarized in Table 3.1) over the older tube gel method (Görg 1989, Görg 1991). Most proteomics labs are choosing IPG technology, and for this reason, only this technique will be discussed here.

Table 3.1. Advantages of IPG strips over tube gels for first-dimension IEF.

ReadyStrip™ IPG Strips	Tube Gels With Carrier Ampholytes
Reproducibility	
Supplied ready to use	Poured by the user
Computer-controlled gradient formation	Ampholyte-based self-forming internal gradients
pH gradient is covalently incorporated into acrylamide matrix and immobilized	pH gradient may drift under conditions of: • Protein overload • Extended run length
Tightly controlled pH gradient and gel length (± 2 mm) for consistent first-dimension separations	Variable gel lengths because of difficulty in casting gel tubes
Ease of Use	
Plastic backing supports acrylamide matrix	Fragile low-percentage acrylamide matrix
Stored in freezer until ready to use	Tube gels cast prior to experiment
Self-centering strips are applied easily to the top of standard second-dimension gels	Gels must be extruded from tubes onto the top of second-dimension gels
Large protein loads can be applied	Limited amount of sample can be applied
Throughput	
Up to twelve 11 cm or 17 cm strips or twenty-four 7 cm strips in each run	Sample loaded at beginning of run
Rehydration/equilibration trays allow for rehydration of one sample while another is being focused	Very difficult and fragile application of tube gel onto second-dimension gel

For methods related to this section see page 32

IPG Strips

A stable, linear, and reproducible pH gradient is crucial to successful IEF. IPG strips offer the advantage of gradient stability over extended focusing runs (Bjellqvist et al. 1982). IPG strips are much more difficult to cast than carrier ampholyte gels (Righetti 1983); however, IPG strips are commercially available, for example as ReadyStrip™ IPG strips. (See Table 3.2 for gradients and sizes currently available.)

pH gradients for IPG strips are created with sets of acrylamido buffers, which are derivatives of acrylamide containing both reactive double bonds and buffering groups. The general structure is CH_2=CH–CO–NH–R, where R contains either a carboxyl [–COOH] or a tertiary amino group (e.g., –$N(CH_3)_2$). These acrylamide derivatives are covalently incorporated into polyacrylamide gels at the time of casting and can form almost any conceivable pH gradient (Righetti 1990).

Choice of pH Gradient Ranges

Use of broad-range strips (pH 3–10) allows the display of most proteins in a single gel. With narrow-range and micro-range overlapping gradient strips, resolution is increased by expanding a small pH range across the entire width of a gel. Since many proteins are focused in the middle of the pH range 3–10, some researchers use nonlinear (NL) gradients to better resolve proteins in the middle of the pH range and to compress the width of the extreme pH ranges at the ends of the gradients. However, overlapping narrow-range and micro-range linear IPG strips can outperform a nonlinear gradient and display more spots per sample (see Figure 3.2). This result is due to the extra resolving power from use of a narrower pI range per gel. Use of overlapping gradients also allows the ability to create "cyber" or composite gels by matching spots from the overlapping regions using imaging software. Because proteins outside of the pH range of the

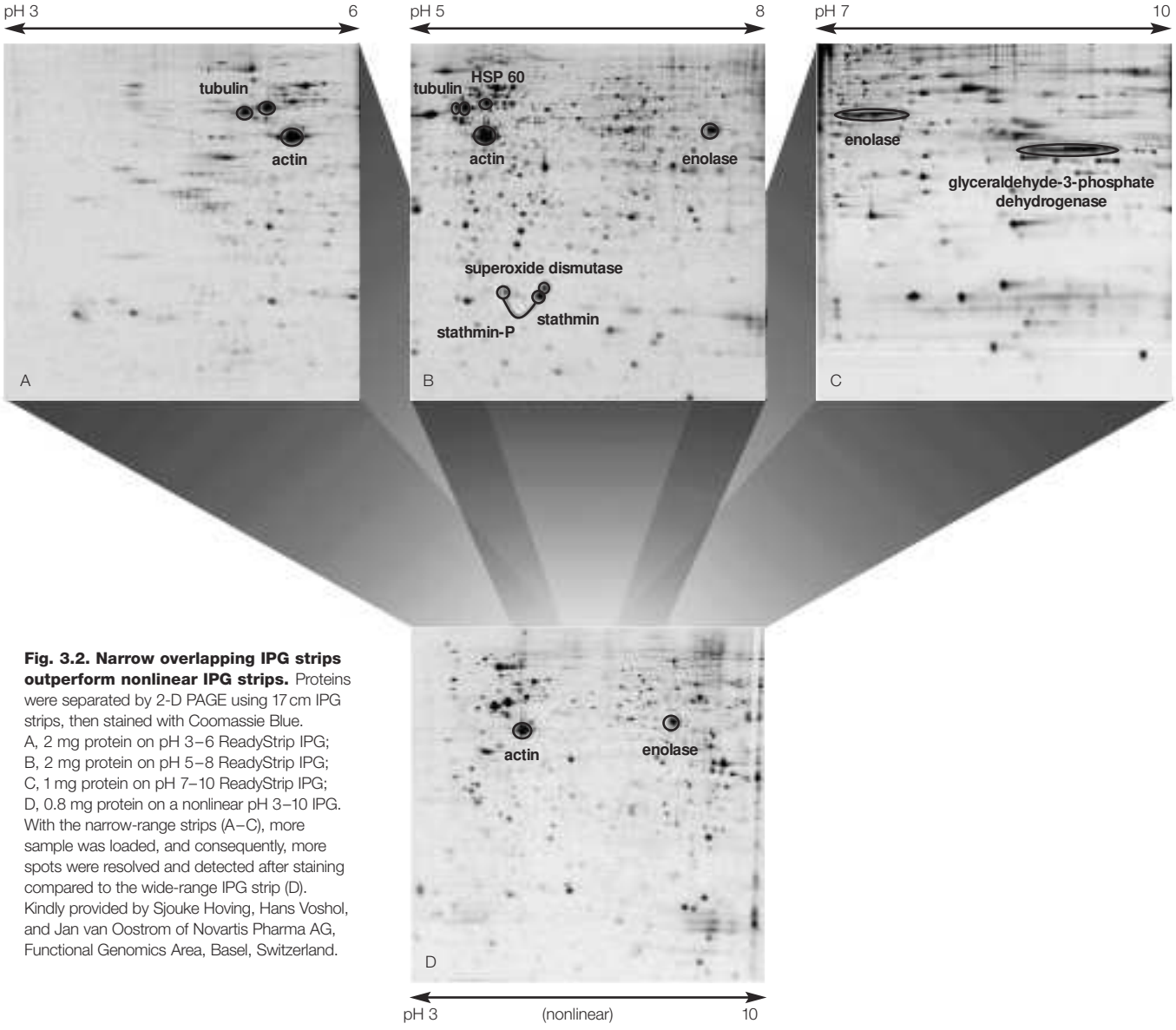

Fig. 3.2. Narrow overlapping IPG strips outperform nonlinear IPG strips. Proteins were separated by 2-D PAGE using 17 cm IPG strips, then stained with Coomassie Blue. A, 2 mg protein on pH 3–6 ReadyStrip IPG; B, 2 mg protein on pH 5–8 ReadyStrip IPG; C, 1 mg protein on pH 7–10 ReadyStrip IPG; D, 0.8 mg protein on a nonlinear pH 3–10 IPG. With the narrow-range strips (A–C), more sample was loaded, and consequently, more spots were resolved and detected after staining compared to the wide-range IPG strip (D). Kindly provided by Sjouke Hoving, Hans Voshol, and Jan van Oostrom of Novartis Pharma AG, Functional Genomics Area, Basel, Switzerland.

For ordering information related to this section see pages 45–46

strip are excluded, more total protein mass can be loaded per strip, allowing more proteins to be detectable. Figure 3.2 demonstrates the resolution achieved using 3 overlapping gradients with 17 cm ReadyStrip IPG strips and PROTEAN® XL gels.

IPG Strip (2-D Array) Size

The 17 cm IPG strips and large-format gels have a large area to resolve protein spots; however, they take a long time to run. Using a mini system instead of, or as a complement to, a large gel format can provide significant time savings. A mini system is perfect for rapid optimization of sample preparation methods. Switching to a large format then allows thorough assessment of a complex sample and identification of proteins of interest. In many cases, a mini system consisting of narrow-range IPG strips can then be used to focus in on the proteins of interest.

Throughput of the 2-D process is a consideration in choosing gel size. Table 4.2 (page 15) compares size formats by experimental run times and equipment available. Table 3.2 shows the appropriate ReadyStrip IPG strip sizes for each of Bio-Rad's second-dimension gel formats. The ability to cast or run 12 gels at a time in any of 3 size formats is very useful in gathering proteomic results. In some cases, mini systems (7 cm ReadyStrip IPG strips with Mini-PROTEAN® 3 format gels, or 11 cm ReadyStrip IPG strips with Criterion™ precast gels) can completely replace large 2-D systems, providing speed, convenience, and ease in handling. The availability of narrow and micro overlapping pH-range ReadyStrip IPG strips can increase the effective width of pI resolution more than 5-fold after accounting for overlapping regions. When 3 narrow-range overlapping ReadyStrip IPG strips are used with the Criterion system, the resolution in the first dimension is increased from 11 to 26 cm. When micro-range strips are used, the resolution in the first dimension is expanded from 11 to 44 cm.

Estimation of pI

The pI of a protein can be estimated by comparing the position of the protein spot of interest to the position of known proteins or standards separated across the same pH gradient (Bjellqvist et al. 1993a, Garfin 2000). ReadyStrip IPG strips contain linear gradients, so the pI of an unknown protein can be estimated by linear interpolation relative to proteins of known pI.

Sample Application

Commercial IPG strips are dehydrated and must be rehydrated to their original gel thickness (0.5 mm) before use. This allows flexibility in applying sample to the strips. There are 3 methods for sample loading: passive in-gel rehydration with sample, active in-gel rehydration with sample, or cup loading of sample after IPG rehydration. Introducing the sample while rehydrating the strips is the easiest, and in most cases the most efficient, way to apply sample. In some specific instances, it is best to rehydrate the strips and then apply sample through sample cups while current is applied. Each method is discussed in the following sections.

Table 3.2. ReadyStrip IPG strips available from Bio-Rad.

17 cm ReadyStrip IPG Strips	Catalog #	Second-Dimension Gel
3 – 10	163-2007	PROTEAN XL, PROTEAN Plus, or PROTEAN Ready Gel® precast gels
3 – 10 (NL)	163-2009	
4 – 7	163-2008	
3 – 6	163-2010	
5 – 8	163-2011	
7 – 10	163-2012	
3.9 – 5.1	163-2020	
4.7 – 5.9	163-2021	
5.5 – 6.7	163-2022	
6.3 – 8.3	163-2023	
11 cm ReadyStrip IPG Strips		
3 – 10	163-2014	Criterion precast gels Gels hand cast in empty Criterion cassettes
3 – 10 (NL)	163-2016	
4 – 7	163-2015	
3 – 6	163-2017	
5 – 8	163-2018	
7 – 10	163-2019	
3.9 – 5.1	163-2024	
4.7 – 5.9	163-2025	
5.5 – 6.7	163-2026	
6.3 – 8.3	163-2027	
7 cm ReadyStrip IPG Strips		
3 – 10	163-2000	Mini-PROTEAN 3 gels Ready Gel precast gels
3 – 10 (NL)	163-2002	
4 – 7	163-2001	
3 – 6	163-2003	
5 – 8	163-2004	
7 – 10	163-2005	
3.9 – 5.1	163-2028	
4.7 – 5.9	163-2029	
5.5 – 6.7	163-2030	
6.3 – 8.3	163-2031	

For methods related to this section see pages 32–33

Sample Application During Rehydration

For both active and passive rehydration methods, the sample is introduced to the IPG strip at the time of rehydration. As the strips hydrate, proteins in the sample are absorbed and distributed over the entire length of the strip (Sanchez et al. 1997).

In the case of active rehydration, a very low voltage is applied during rehydration of the strips. Proteins enter the gel matrix under current as well as by absorption. The PROTEAN IEF cell has preprogrammed methods designed to accommodate active rehydration (see page 33 for the protocol). Active rehydration is thought to help large proteins enter the strip by applying electrical "pull". Because the voltage is applied before all the solution and proteins are absorbed into the gel, the pH of a protein's environment will be the pH of the rehydration buffer, and the protein will move according to its mass-to-charge ratio in that environment. Thus, small proteins with a higher mobility have a higher risk of being lost from the strip.

With passive rehydration, proteins enter the gel by absorption only (see page 30 for the protocol). This method allows efficient use of equipment since strips can be rehydrated in sample rehydration trays while other samples are being focused in the IEF cell.

Whether the strips are hydrated actively or passively, it is very important that they be incubated with sample for at least 11 hr prior to focusing. This allows the high molecular weight proteins time to enter the gel after the gel has become fully hydrated and the pores have attained full size. These sample application methods work because IEF is a steady-state technique, so proteins migrate to their pI independent of their initial positions.

The advantages of this approach are:

- Sample application is simple (Görg et al. 1999)
- Sample application during rehydration avoids the problem of sample precipitation, which often occurs with cup loading (Rabilloud 1999)
- Shorter focusing times can be used because the sample proteins are in the IPG strip prior to IEF
- Very large amounts of protein can be loaded using this method

Sample Application by Cup Loading

Cup loading can be beneficial in the following cases (Cordwell et al. 1997, Görg et al. 2000):

- When samples contain high levels of DNA, RNA, or other large molecules, such as cellulose
- For analytical serum samples that have not been treated to remove albumin
- When running basic IPG strips; e.g., pH 7–10
- For samples that contain high concentrations of glycoproteins

Because of its relative difficulty and tendency toward artifacts, cup loading should be avoided if possible. When loading the protein sample from a cup, the IPG strips must be rehydrated prior to sample application. The IPG strips can be rehydrated in a variety of ways. We recommend the rehydration tray, although IPG strips are often rehydrated in 1 or 2 ml pipets that have been sealed at both ends with Parafilm. Sample volumes of up to 100 µl can be loaded later onto each gel strip using a sample cup.

Power Conditions and Resolution in IEF

During an IEF run, the electrical conductivity of the gel changes with time, especially during the early phase. When an electrical field is applied to an IPG at the beginning of an IEF run, the current will be relatively high because of the large number of charge carriers present. As the proteins and ampholytes move toward their pIs, the current will gradually decrease due to the decrease in the charge on individual proteins and carrier ampholytes.

The pH gradient, strip length, and the applied electrical field determine the resolution of an IEF run. According to both theory and experiment, the difference in pI between two adjacent IEF-resolved protein bands is directly proportional to the square root of the pH gradient and inversely proportional to the square root of the voltage gradient at the position of the bands (Garfin 2000).

Thus, narrow pH ranges and high applied voltages yield high resolution in IEF. The highest resolution can be achieved using micro-range IPG strips and an electrophoretic cell, such as the PROTEAN IEF cell, capable of applying high voltages. IEF runs should always be carried out at the highest voltage compatible with the IPG strips and electrophoretic cell. However, high voltages in electrophoresis are accompanied by large amounts of generated heat. The magnitude of the electric field that can be applied and the ionic strength of the solutions that can be used in IEF are limited. Thin gels are better able to dissipate heat than thick ones and are therefore capable of withstanding the high voltage that leads to higher resolution. Also, at the completion of focusing, the current drops to nearly zero since the carriers of the current have stopped moving. The PROTEAN IEF cell is designed to provide precise cooling, allowing the highest possible voltages to be applied. (A default current limit of 50 µA per strip is intended to minimize protein carbamylation reactions in urea sample buffers. This limit can be increased to 99 µA per strip.)

For ordering information related to this section see pages 45–46

Table 3.3. The time to reach programmed volt-hours varies with the pH gradient.

Strip Size; V-hr Programmed	pH 3–10	pH 3–6	pH 4–7	pH 5–8	pH 7–10
7 cm, 8,000 V-hr	2 hr 30 min	3 hr 45 min	2 hr	1 hr 30 min	2 hr
11 cm, 20,000 V-hr	4 hr 50 min	6 hr 30 min	3 hr 45 min	3 hr 45 min	3 hr 45 min
17 cm, 50,000 V-hr	7 hr 20 min	9 hr	6 hr 30 min	6 hr	6 hr 30 min

Average amount of time for the PROTEAN IEF cell to achieve the programmed volt-hours. The time required varied by up to 20% between trials. Each strip contained the same *E. coli* sample in the same rehydration solution. The current was limited to 50 µA per strip using a rapid ramp. The time is extended when the voltage is limited by high current. In most cases, the voltage never reaches the maximum voltage set.

Consistent and reproducible focusing requires that the time integral of voltage (volt-hours) be kept consistent. It is usually necessary to program IEF runs to reach final focusing voltages in stages. This approach clears ionic constituents in the sample from the IPG strips while limiting electrical heating of the strips. The PROTEAN IEF cell allows for multistep runs at durations set by time or volt-hours. Some suggested starting electrical conditions and voltage ramping options are discussed on page 34.

The number of volt-hours required to complete a run must be determined empirically. A simple *E. coli* extract can yield equally acceptable 2-D results when 7 cm strips are run for either 8,000 V-hr or 22,000 V-hr, when 11 cm strips are run for either 20,000 V-hr or 41,500 V-hr, or when 17 cm strips are run for either 50,000 V-hr or 85,600 V-hr. This result is sample- and buffer-dependent. A more complex sample in terms of number of proteins or even a different sample buffer might require increased volt-hours.

The time needed to achieve the programmed volt-hours depends on the pH range of the IPG strip used as well as sample and buffer characteristics. Table 3.3 shows the variability in time required to run the same sample and rehydration solution on different strip sizes and pH gradients. These data support running similar strips and samples in batches. If different strips are run at the same time, the electrical conditions experienced by individual strips will be different, perhaps exposing some strips to more current than desired, since the total current limit is averaged over all strips in a tray.

For methods related to this section see page 34

Chapter 4 — The Second Dimension: SDS-PAGE

Protein Separation by Molecular Weight (MW)

Second-dimension separation is by protein mass, or MW, using SDS-PAGE. The proteins resolved in IPG strips in the first dimension are applied to second-dimension gels and separated by MW perpendicularly to the first dimension.

The pores of the second-dimension gel sieve proteins according to size because dodecyl sulfate coats all proteins essentially in proportion to their mass. The net effect is that proteins migrate as ellipsoids with a uniform negative charge-to-mass ratio, with mobility related logarithmically to mass (Garfin 1995). See Figure 4.1.

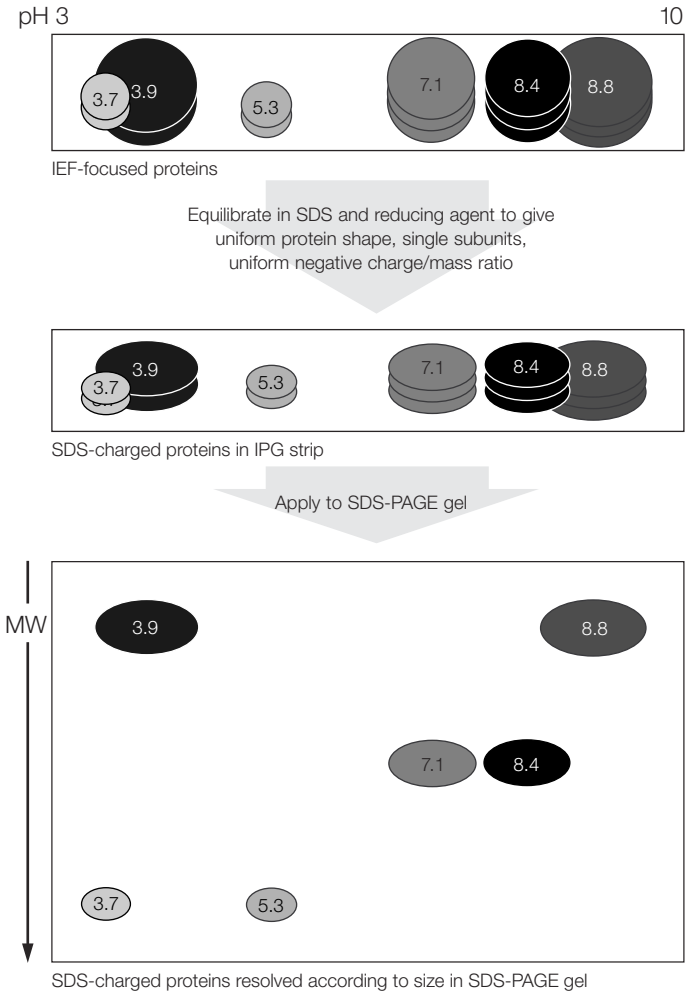

Fig. 4.1. Schematic diagram showing separation of proteins by SDS-PAGE after separation by IEF.

Gel Composition

Homogeneous (single-percentage acrylamide) gels generally give excellent resolution of sample proteins that fall within a narrow MW range. Gradient gels have two advantages: they allow proteins with a wide range of MW to be analyzed simultaneously, and the decreasing pore size along the gradient functions to sharpen the spots.

Single-Percentage Gels

The percentage of acrylamide, often referred to as %T (total percentage of acrylamide plus crosslinker) determines the pore size of a gel. Most protein separations use 37.5 parts acrylamide to 1 part bis-acrylamide (bis). Some researchers substitute piperazine diacrylamide (PDA) for bis, which can reduce silver staining background and give higher gel strength. The higher the %T, the smaller the pore size. A suitable %T can be estimated from charts of mobility for proteins of different MW. Table 4.1 shows the MW ranges resolved on gels of different acrylamide percentages.

Gradient Gels

Gradient gels are cast with acrylamide concentrations that increase from top to bottom so that the pore size decreases as proteins migrate further into the gels. As proteins move through gradient gels from regions of relatively large pores to regions of relatively small pores, their migration rates slow. Small proteins remain in gradient gels much longer than they do in single-percentage gels that have the same average %T, so both large and small molecules may be resolved in the same gel. This makes gradient gels popular for analysis of complex mixtures that span wide MW ranges. A gradient gel, however, cannot match the resolution obtainable with a properly chosen single concentration of acrylamide. A good approach is to use gradient gels for estimates of the complexities of mixtures. A proteomics experiment might start out with an 8–16%T gradient for global comparison. After interesting regions of the 2-D array have been identified, a new set of single-percentage gels may be run to study a particular size range of proteins.

It is simplest and often most cost and labor effective to purchase commercially available precast gradient gels. Bio-Rad offers a full line of gels for 2-D PAGE (Table 4.1) as well as devices to cast gels of different sizes in multi-casting chambers (Table 4.2).

For ordering information related to this section see page 46

Precast Gels

High-quality precast gels are preferred for high-throughput applications. They provide savings in time and labor, and the precision-poured gradients result in reproducibility among runs. Bio-Rad offers precast gels with IPG wells in three size formats to hold three lengths of ReadyStrip™ IPG strips. A full list of the gels currently available with IPG wells can be found in Table 4.1.

Precast gels differ from handcast gels in that they are cast with a single buffer throughout and without SDS. During storage, different buffers in the stacking and resolving gels would mingle without elaborate means to keep them separate, and thus have no practical value. Also, because the sample contains SDS, and the dodecyl sulfate ion in the cathode buffer moves faster than the proteins in the gel, keeping them saturated with the detergent, precast gels are made without SDS.

Choosing a Size Format

Bio-Rad offers complete second-dimension systems for three gel sizes as detailed in Table 4.2. Mini-PROTEAN® 3, Criterion™, and PROTEAN® XL formats have precast gels available. A fourth PROTEAN Plus™ size is available to run in the PROTEAN Plus Dodeca™ cell and can be cast as 20 x 20.5 cm gels or as 25 x 20.5 cm gels. The large gel can hold one 17 cm or two 11 cm ReadyStrip IPG strips.

The large-format PROTEAN Ready Gel® precast gels may be run in either the PROTEAN XL cell (2 gels per run), the PROTEAN XL multi-cell (6 gels per run), or the PROTEAN Plus Dodeca cell (12 gels per run). The Criterion precast gels can be run in the Criterion cell (2 gels per run) or the Criterion Dodeca cell (up to 12 gels per run). Mini-PROTEAN 3 or Ready Gel precast gels can be run in the Mini-PROTEAN 3 cell (2 gels per run) or the Mini-PROTEAN 3 Dodeca cell (up to 12 gels per run).

Transition From First to Second Dimension

The transition from first-dimension to second-dimension gel electrophoresis involves two steps: equilibration of the resolved IPG strips in SDS reducing buffer, and embedding of the strip on the top of the second-dimension gel. Proper equilibration simultaneously ensures that proteins are coated with dodecyl sulfate and that cysteines are reduced and alkylated. A method for IPG strip equilibration is discussed on page 36. The equilibrated IPG strips are placed on top of the gel and fixed with molten agarose solution to ensure good contact between the gel and the strip.

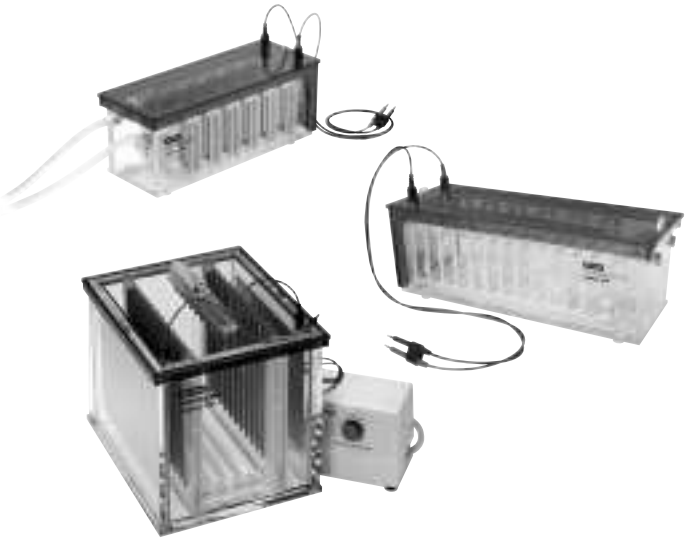

Fig. 4.2. The Dodeca cells. Clockwise from upper left: Mini-PROTEAN 3 Dodeca cell, Criterion Dodeca cell, and PROTEAN Plus Dodeca cell.

Table 4.1. Second-dimension precast gels from Bio-Rad.

Gel Format	Catalog #	Composition	Protein Mass Range Resolved
PROTEAN II Ready Gel Precast Gels	161-1450	10%	30–150 kD
Tris-HCl, IPG well	161-1451	12%	20–100 kD
(For use with 17 cm ReadyStrip IPG strips;	161-1452	10–20%	10–100 kD
gel size 18.3 x 19.3 cm)	161-1453	8–16%	10–100 kD
	345-0013	10% resolving, 4% stacking gel	30–150 kD
Criterion Precast Gels	345-0018	12.5% resolving, 4% stacking gel	20–100 kD
Tris-HCl, IPG well	345-0031	4–15%	20–250 kD
(For use with 11 cm ReadyStrip IPG strips;	345-0036	4–20%	10–200 kD
gel size 13.3 x 8.7 cm)	345-0041	8–16%, 4% stacking gel	10–100 kD
	345-0046	10–20%, 4% stacking gel	10–100 kD
	161-1390	10% resolving, 4% stacking gel	30–150 kD
Ready Gel Precast Gels	161-1391	12% resolving, 4% stacking gel	20–100 kD
Tris-HCl, IPG well	161-1392	4–15%	20–250 kD
(For use with 7 cm ReadyStrip IPG strips;	161-1393	4–20%	10–200 kD
gel size 7.4 x 6.8 cm)	161-1394	8–16% resolving, 4% stacking gel	10–100 kD
	161-1395	10–20% resolving, 4% stacking gel	10–100 kD

For methods related to this section see page 35

Table 4.2. Size formats for second-dimension electrophoresis.

	Mini-PROTEAN 3 System	Criterion System	PROTEAN II XL System	PROTEAN Plus Dodeca Cell System
ReadyStrip IPG strip	7 cm	11 cm	17 cm	17 cm or 2 x 11 cm or 3 x 7 cm
Precast second-dimension gel (W x L)	Ready Gel precast gels 7.4 x 6.8 cm	Criterion precast gels 13.3 x 8.7 cm	PROTEAN II Ready Gel precast gels 18.3 x 19.3 cm	PROTEAN II Ready Gel precast gels 18.3 x 19.3 cm
Handcast second-dimension gel (W x L)	Mini-PROTEAN 3 handcast gels 7.4 x 6.8 cm	Criterion empty cassettes 13.3 x 8.7 cm	PROTEAN II XL handcast gels 18.3 x 19.3 cm	PROTEAN Plus handcast gels 20 x 20.5, 25 x 20.5 cm
Cell formats available	Mini-PROTEAN 3 cell runs 2 gels Mini-PROTEAN 3 Dodeca cell runs 12 gels	Criterion cell runs 2 gels Criterion Dodeca cell runs 12 gels	PROTEAN II XL cell runs 2 gels PROTEAN II XL multi-cell runs 6 gels	PROTEAN Plus Dodeca cell runs 12 gels
Second-dimension run time	35 min	1 hr	5–6 hr	5–6 hr
Total electrophoresis run time*	5.5–7.5 hr	5.75–8 hr	13–15 hr	13–15 hr

* Does not include 12 hr rehydration or 30 min equilibration time for strips, or staining time for gels.

Second Dimension and High Throughput

Since the first dimension can be run in batches of 12–24 strips at a time, it is desirable to run the same number of samples in the second dimension. Precast gels ensure high reproducibility among samples and help reduce the work involved in running large numbers of samples. Alternatively, gels can be hand cast 12 at a time under identical conditions with multi-casting chambers. The Dodeca cells (Figure 4.2) save time, space, and effort, and help to ensure that gels are run under the same electrical conditions for highest throughput and reproducibility.

MW Estimation

The migration rate of a polypeptide in SDS-PAGE is inversely proportional to the logarithm of its MW. The larger the polypeptide, the more slowly it migrates in a gel. MW is determined in SDS-PAGE by comparing the migration of protein spots to the migration of standards. Plots of log MW versus the migration distance are reasonably linear. Gradient SDS-PAGE gels can also be used to estimate MW. In this case, log MW is proportional to log (%T). With linear gradients, %T is proportional to distance migrated, so the data can be plotted as log MW vs. log (migration distance).

Standard curves are actually sigmoid. The apparent linearity of a standard curve may not cover the full MW range for a given protein mixture in a particular gel. However, log MW varies sufficiently slowly to allow fairly accurate MW estimates to be made by interpolation, and even extrapolation, over relatively wide ranges (Garfin 1995).

Mixtures of standard proteins with known MW are available from Bio-Rad in several formats for calibrating the migration of proteins in electrophoretic gels. Standards are available unstained, prestained, or with tags for development with various secondary reagents (useful when blotting). Standards can be run in a reference well, attached to the end of a focused IPG strip by filter paper, or directly embedded in agarose onto the second-dimension gel (see method on page 37).

For ordering information related to this section see pages 46–48

Chapter 5 — Detection of Proteins in Gels

Guidelines for Detection of Proteins in Gels

Gels are run for either analytical or preparative purposes. The intended use of the gel determines the amount of protein to load and the means of detection. It is most common to make proteins in gels visible by staining them with dyes or metals. Each type of protein stain has its own characteristics and limitations with regard to the sensitivity of detection and the types of proteins that stain best (see Table 5.1). Sometimes proteins are transferred to membranes by western blotting to be detected by immunoblotting, glycoprotein analysis, or total protein stain (see Chapter 6).

If the purpose of gel electrophoresis is to identify low-abundance proteins (e.g., low-copy-number proteins in a cell extract, or contaminants in a purification scheme), then a high protein load (0.1–1 mg/ml) and a high-sensitivity stain, such as silver or a fluorescent stain, should be used (Corthals et al. 2000). When the intention is to obtain enough protein for use as an antigen or for sequence analysis, then a high protein load should be applied to the gel and the proteins visualized with a staining procedure that does not fix proteins in the gel. Quantitative comparisons require the use of stains with broad linear ranges of detection.

The sensitivity that is achievable in staining is determined by: 1) the amount of stain that binds to the proteins; 2) the intensity of the coloration; 3) the difference in coloration between stained proteins and the residual background in the body of the gel (the signal-to-noise ratio). Unbound stain molecules can be washed out of the gels without removing much stain from the proteins.

All stains interact differently with different proteins (Carroll et al. 2000). No stain will universally stain all proteins in a gel in proportion to their mass. The only observation that seems to hold for most stains is that they interact best with basic amino acids. For critical analysis, replicate gels should be stained with two or more different stains. Of all stains available, colloidal Coomassie Blue (Bio-Safe™ Coomassie) appears to stain the broadest spectrum of proteins. It is instructive, especially with 2-D PAGE gels, to stain a colloidal Coomassie Blue-stained gel with silver or to stain a fluorescently stained gel with colloidal Coomassie Blue or silver. Very often, this double staining procedure will show a few differences between the protein patterns. It is most common to stain gels first with Coomassie Blue or a fluorescent stain, then restain with silver. However, the order in which the stains are used does not seem to be important, as long as the gels are washed well with high-purity water between stains.

Coomassie Blue Staining

Coomassie Brilliant Blue R-250 is the most common stain for protein detection in polyacrylamide gels. Coomassie Brilliant Blue R-250 and G-250 are wool dyes that have been adapted to stain proteins in gels. The “R” and “G” designations indicate red and green hues, respectively.

Coomassie R-250 requires on the order of 40 ng of protein per spot for detection. Absolute sensitivity and staining linearity depend on the proteins being stained. The staining solution also fixes most proteins in gels.

Bio-Safe Coomassie stain is made with Coomassie Brilliant Blue G-250. Bio-Safe Coomassie stain is a ready-to-use, single-reagent protein stain. Sensitivity can be down to 10 ng, and greater contrast is achieved by washing the gel in water after staining. Used stain can be disposed of as nonhazardous waste and the procedure does not fix proteins in the gel.

SYPRO Ruby Fluorescent Staining

SYPRO Ruby protein gel stain has desirable features that make it popular in high-throughput laboratories. It is an endpoint stain with little background staining (high signal-to-noise characteristics) and it is sensitive and easy to use. SYPRO Ruby protein stain does not detect nucleic acids.

SYPRO Ruby protein stain is sensitive to 1–10 ng and can be linear over 3 orders of magnitude. It is compatible with high-throughput protocols and downstream analysis, including mass spectrometry and Edman sequencing (Patton 2000). It also allows detection of glycoproteins, lipoproteins, low MW proteins, and metalloproteins that are not stained well by other stains. This fluorescent stain is easily visualized with simple UV or blue-light transilluminators, as well as by the Molecular Imager FX™ Pro Plus multiimager and VersaDoc™ imaging systems (see pages 21–22).

Table 5.1. Characteristics of protein stains.

Gel Stain	Sensitivity	Process Time/# Steps	Advantages
SYPRO Ruby protein gel stain	1 ng	3 hr/2 steps	Mass spectrometry compatible; linear over 3 orders of magnitude; allows protein analysis in fluorescent imagers
Bio-Safe Coomassie stain	10 ng	2.5 hr/3 steps	Mass spectrometry compatible; easily visualized; nonhazardous
Silver Stain Plus™ stain	1 ng	1.5 hr/3 steps	Mass spectrometry compatible; high sensitivity; low background
Bio-Rad silver stain	1 ng	2 hr/7 steps	High sensitivity; detects some highly glycosylated and other difficult-to-stain proteins
Coomassie Blue R-250	40 ng	2.5 hr/2 steps	Oldest and least expensive method

For methods related to this section see page 38

Silver Staining

Two popular methods for silver staining are recommended for 2-D analysis. They are based on slightly different chemistries but have similar sensitivities for protein. Bio-Rad's silver stain kit, based on the method of Merril et al. (1981), can be as much as 100 times more sensitive than Coomassie Blue R-250 dye staining and allows visualization of heavily glycosylated proteins in gels. Protein spots containing 10–100 ng of protein can be easily seen. Proteins in gels are fixed with alcohol and acetic acid, then oxidized in a solution of potassium dichromate in dilute nitric acid, washed with water, and treated with silver nitrate solution. Silver ions bind to the oxidized proteins and are subsequently reduced to metallic silver by treatment with alkaline formaldehyde. Color development is stopped with acetic acid when the desired staining intensity has been achieved. This method is not compatible with mass spectroscopic analysis since the oxidative step changes protein mass. See page 39 for more information on the method.

The Silver Stain Plus stain from Bio-Rad requires only one simultaneous staining and development step and is based on the method developed by Gottlieb and Chavko (1987). Proteins are fixed with a solution containing methanol, acetic acid, and glycerol, and washed extensively with water. The gels are then soaked in a solution containing a silver-ammine complex bound to colloidal tungstosilicic acid. Silver ions transfer from the tungstosilicic acid to the proteins in the gel by means of an ion exchange or electrophilic process. Formaldehyde in the alkaline solution reduces the silver ions to metallic silver to produce the images of protein spots. The reaction is stopped with acetic acid when the desired intensity has been achieved. Because silver ions do not accumulate in the bodies of gels, background staining is light. Since this method lacks an oxidizing step, visualization of heavily glycosylated proteins and lipoproteins can be less sensitive than with the Merril stain. This method is better for use in proteomics when the end goal is identification by mass spectrometric analysis. See page 39 for more information on the method.

For ordering information related to this section see page 49

Chapter 6 — Detection of Proteins on Western Blots

Certain synthetic membranes bind proteins tightly enough that they can be used as supports for solid-phase immunoassays, staining, or other solid-phase analysis. These membranes are collectively known as western blots (Bers and Garfin 1985, Garfin and Bers 1989, Ledue and Garfin 1997). Bound proteins retain their antigenicity and are accessible to probes. Several techniques have been developed to probe proteins bound to synthetic membranes.

Apparatus for Blotting

A protein is electrotransferred from a gel to an adjoining membrane by directing an electric field across the gel. There are two types of apparatus for electrotransfer, buffer-filled tanks and semi-dry transfer devices.

Transfer tanks are made of plastic with twoelectrodes mounted near opposing tank walls. A nonconducting cassette holds the membrane in close contact with the gel. The cassette assembly is placed vertically into the tank, parallel to the electrodes, and submerged in electrophoresis buffer. A large volume of buffer in the tank dissipates the heat generated during transfer. Table 6.1 lists specifications for tank blotters.

In semi-dry blotting, the gel and membrane are sandwiched horizontally between two stacks of buffer-wetted filter paper in direct contact with two closely spaced solid plate electrodes. The close spacing of the semi-dry apparatus provides high field strengths. The term “semi-dry” refers to the limited amount of buffer, which is confined to the stacks of filter paper. See Table 6.2 for Bio-Rad’s Trans-Blot® SD semi-dry blotter specifications.

With tanks, transfers are somewhat more efficient than with semi-dry devices. Under semi-dry electrotransfer conditions, some low MW proteins are driven through the membranes, and because low buffer capacity limits run times, some high MW proteins are poorly transferred. Conversely, the liquid in tank blotters can be efficiently cooled, allowing slower transfers without heat buildup. Slower transfer conditions can allow the time needed for large proteins to move through the gel matrix, but the lower intensity allows small proteins to remain attached to the membrane after transfer.

Membranes and Buffers for Immunoblotting

The two membranes most used for protein immunoblotting work are nitrocellulose and polyvinylidene fluoride (PVDF). Both bind proteins at about 100–200 µg/cm^2. In addition, PVDF can be used when proteins are to be sequenced. It can withstand the harsh chemicals of protein sequencers, whereas nitrocellulose cannot.

Table 6.1. Bio-Rad’s tank blotters.

	Mini Trans-Blot® Cell	Criterion™ Blotter	Trans-Blot Cell
Number of gels per run	• 2 Mini-PROTEAN® 3 gels	• 2 Criterion gels • 4 Mini-PROTEAN 3 gels	**Low intensity** • 3 PROTEAN® II XL gels • 6 Criterion gels • 12 Mini-PROTEAN 3 gels **High intensity** • 1 PROTEAN II XL gel • 2 Criterion gels • 4 Mini-PROTEAN 3 gels
Buffer volume	450 ml	1.3 L	2.5 L
Electrode material	Wire	Wire or plate	Wire or plate
Electrode distance	4 cm	4.3 cm	8 cm or 4 cm
Time	1 hr	30 min to overnight	30 min to overnight
Temperature control	Bio-Ice™ block	Sealed ice block or cooling coil	Cooling coil

Table 6.2. Bio-Rad’s Trans-Blot SD semi-dry blotter.

Blotter	Number of Gels/Run (single layer, more can be run in stacks)	Electrode Material and Surface Area	Highlights
Trans-Blot SD	1 PROTEAN II XL gel 6 Mini-PROTEAN 3 gels 3 Criterion gels	Platinum-coated titanium anode Stainless-steel cathode (18 x 25 cm)	Durable electrode pair with optimal electrochemical properties Spring-loaded electrodes give uniform pressure for consistent results Easy to assemble and use Voltage limit of 25 V

For ordering information related to this section see pages 49–50

Immunoblotting

Development of immunoblots will range in complexity, depending on the method. Figure 6.1 illustrates three methods of development; each is available as a kit from Bio-Rad. The general procedure is summarized below:

1. Proteins are transferred from a 2-D gel to a membrane surface. The transferred proteins become immobilized on the surface of the membrane in a pattern that is an exact replica of the gel.
2. Unoccupied protein-binding sites on the membrane are saturated to prevent nonspecific binding of antibodies. This step is called either blocking or quenching.
3. The blot is probed for the protein of interest with a specific primary antibody.
4. The blot is probed a second time. The second probe is an antibody that is specific for the primary antibody type and is conjugated to a detectable enzyme. The site of the protein of interest is thus tagged with an enzyme through the specificities of the primary and secondary antibodies.
5. Enzyme substrates that are converted into detectable products are incubated with the blot. The enzyme products indicate the positions of the proteins of interest.

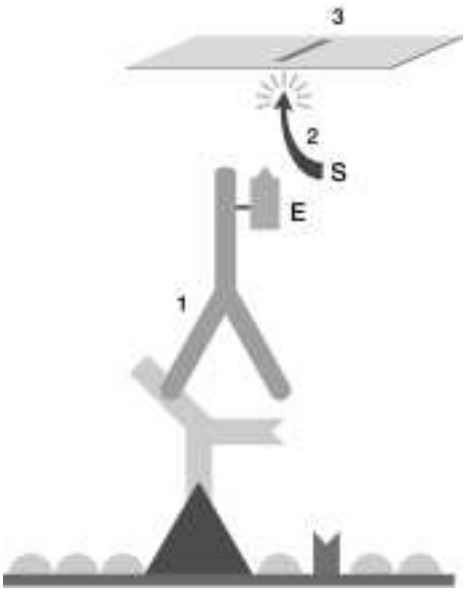

Fig. 6.1 B. Immun-Star™ chemiluminescent detection kit.
1. Alkaline phosphatase (AP)-conjugated secondary antibody binds to primary antibody
2. Chemiluminescent substrate reacts with AP to emit light
3. Film or phosphor screen is exposed to emitted light

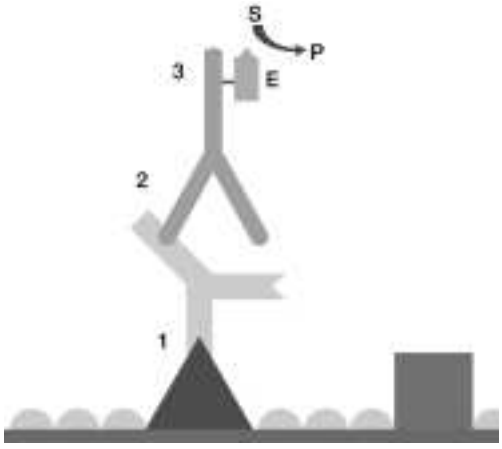

Fig. 6.1 A. General color detection system.
1. Antigen-specific primary antibody binds to protein of interest
2. Enzyme-conjugated secondary antibody or binding protein binds to primary antibody
3. Enzyme (E) converts added substrate (S) to colored product (P), which precipitates onto the membrane surface

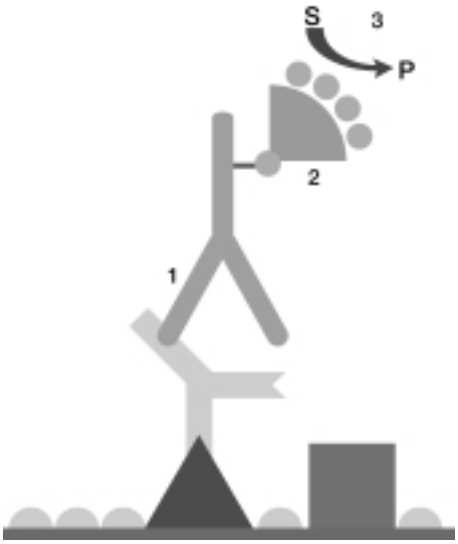

Fig. 6.1 C. Amplified AP Immun-Blot® kit.
1. Biotinylated secondary antibody binds to primary antibody
2. Complex of streptavidin and biotinylated AP binds to biotin of secondary antibody
3. Multiple AP molecules are now available to convert substrate (S) to colored product (P), which precipitates

For ordering information related to this section see pages 49–50

On-Membrane Detection of Glycoproteins after 2-D Electrophoresis

Eukaryotic proteins often appear on 2-D PAGE gels as “trains” of spots that differ in apparent pI, MW, or both. These are usually isoforms of the same protein and result from a variety of posttranslational modifications, including glycosylation.

The initial step, once a blot has been prepared, is to identify which spots are glycoproteins so that they can be further characterized. Various methods have been developed for the detection of glycoproteins on 2-D gels and blots by color and lectin analysis, and these can be carried out at the analytical level. The actual level of detection of course depends on the extent of glycosylation of the protein, since the reagents react only with the carbohydrate moiety.

The Immun-Blot glycoprotein detection kit is based on the initial oxidation of the carbohydrate with periodic acid. Periodic acid oxidizes vicinal diols on terminal monosaccharides to dialdehydes. Biotin hydrazide is coupled to these aldehyde groups. The biotinylated glycoproteins are then detected by coupling to streptavidin-alkaline phosphatase (AP) followed by reaction with a color-development substrate system (Figure 6.2). The limit of detection by this method is about 0.5 µg of glycoprotein containing a single N-linked oligosaccharide.

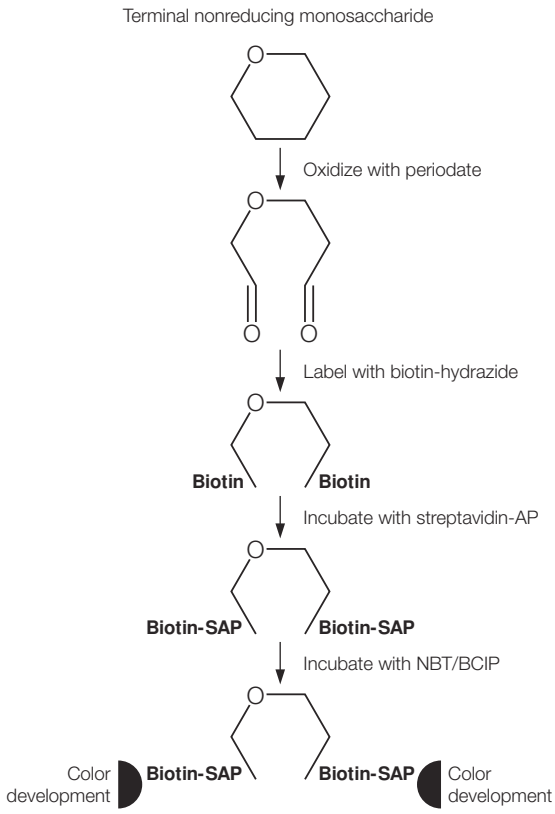

Fig. 6.2. Schematic illustration of glycoprotein detection chemistry using Bio-Rad’s Immun-Blot glycoprotein detection kit.

Total Protein Detection on Blots

For proper identification of the proteins of interest in a blot, immunodetected proteins or glycoproteins must be compared to the total protein pattern of the gel. This requires the indiscriminate staining of all the proteins in the blot. Colloidal gold stain is a very sensitive reagent for total protein staining. It is a stabilized solution of colloidal gold particles. The gold particles bind to proteins on the surfaces of membranes. Detection limits are in the 100 pg range and can be enhanced an order of magnitude by subsequent treatment with silver (Dunn 1999).

Coomassie Blue R-250 and Bio-Safe™ Coomassie stains are other popular total protein stains. Researchers blotting 2-D PAGE gels particularly favor them since they are compatible with mass spectrometry. Stained blots are well suited for the archiving of 2-D PAGE separations. SYPRO Ruby protein blot stain is a very sensitive total protein stain that is formulated for blots.

For ordering information related to this section see pages 49–50

Chapter 7 — Image Acquisition and Analysis

Image Acquisition Instruments

Before 2-D gels can be analyzed with an image evaluation system, they must be digitized. The most commonly used devices are camera systems, densitometers, phosphor imagers, and fluorescence scanners. All of Bio-Rad's imaging systems (Table 7.1) are seamlessly integrated with PDQuest™ software, and they can export and import images to and from other software via TIFF files.

Densitometry

Densitometers compare the intensity of a light beam before and after attenuation by a sample.

The GS-800™ calibrated imaging densitometer (Figure 7.1) has been customized for analysis of gels, autoradiograms, and blots. The transmittance and true reflectance capabilities allow accurate scans of samples that are either transparent (gels and film) or opaque (blots). It provides high-quality imaging to resolve close spots and a variable resolution feature to preview and crop images. Wet 2-D gels may be scanned with red, green, and blue color CCD technology on the watertight platen.

Fig. 7.1. GS-800 calibrated imaging densitometer.

Storage Phosphor and Fluorescence Scanners

Digitization of 2-D gels stained with fluorescent dyes or radioactive compounds requires specific imaging systems (Patton 2000).

The Molecular Imager FX™ Pro Plus system (Figure 7.2) is flexible and expandable. 2-D gels of radiolabled proteins can be imaged using a Kodak phosphor screen more rapidly and accurately than with film. Popular proteomic fluorescent stains, including SYPRO Ruby protein gel and blot stains and SYPRO Orange protein gel stain can be imaged with single-color and multicolor fluorescence via direct laser excitation. This system permits detection of almost any fluorophore that is excited in the visible spectrum. The internal laser and external laser options allow optimal excitation of single-color or multicolor fluorescent samples. Computer-controlled, user-accessible filter wheels have eight filter slots, allowing detection of many multicolor combinations of dyes (e.g., Gingrich et al. 2000).

Fig. 7.2. Molecular Imager FX Pro Plus multiimager system.

Table 7.1 Bio-Rad imaging systems.

Instrument	Detection	Detects
Molecular Imager FX Pro Plus multiimager	Isotopic, fluorescent	• SYPRO Ruby protein stains (for gels or blots) • SYPRO Orange protein gel stain • Radioisotopes
Molecular Imager FX™ Pro fluorescent imager	Fluorescent	• SYPRO Ruby protein stains (for gels or blots) • SYPRO Orange protein gel stain
VersaDoc™ Model 5000 imaging system	Fluorescent, chemiluminescent, colorimetric	• SYPRO Ruby protein stains (for gels or blots) • SYPRO Orange protein gel stain • Autoradiographs • Silver, Coomassie R-250, or Bio-Safe™ Coomassie stain
VersaDoc Model 3000 imaging system	Fluorescent, chemiluminescent, colorimetric	• SYPRO Ruby protein stains (for gels or blots) • SYPRO Orange protein gel stain • Autoradiographs • Silver, Coomassie R-250, or Bio-Safe Coomassie stain
GS-800 calibrated imaging densitometer	Colorimetric, isotopic (on film)	• Coomassie R-250 or Bio-Safe Coomassie stain • Silver stain • Autoradiographs

For ordering information related to this section see page 50

The Molecular Imager FX™ Pro system has all the featues of the FX Pro Plus for fluorescent dectection, but without the storage phosphor option. The VersaDoc model 1000, model 3000, and model 5000 imaging systems are able to produce exceptionally high-quality images from single-color or multicolor fluorescent sources, including samples stained with SYPRO Ruby protein gel and blot stains and SYPRO Orange protein gel stain. Chemiluminescent western blots can be detected without the use of film. Gels stained with Coomassie dyes or silver stains as well as autoradiographs can be imaged by densitometry or with a multiimaging system.

Computer-Assisted Image Analysis of 2-D Electrophoretic Gels

Computer-assisted image analysis software is an indispensable tool for the evaluation of complex 2-D gels. It allows:

- Storage and structuring of large amounts of collected experimental image data
- Rapid and sophisticated analysis of experimental information
- Supplementation and distribution of data among labs
- Establishment of 2-D-protein data banks

Image analysis systems deliver error-fee comprehensive qualitative and quantitative data from a large number of 2-D gels (Miller 1989).

PDQuest software from Bio-Rad is a popular analysis tool. Gel analysis of digitized gel images includes spot detection, spot quantitation, gel comparison, and statistical analysis. PDQuest software has the further advantage of seamless integration with any of Bio-Rad's image acquisition instruments, as well as the ability to control the ProteomeWorks™ spot cutter described in Chapter 8 (see Figures 8.1 and 8.2). The advanced annotation feature can be used to label spots with text, URL links, document links, or mass spectrometry data.

Spot Detection and Spot Quantitation

Before the software automatically detects the protein spots of a 2-D gel, the raw image data are corrected and the gel background is subtracted. The process is executed with simple menus and "wizards".

PDQuest software models protein spots mathematically as 3-D Gaussian distributions and uses the models to determine absorption maxima. This enables automatic detection and resolution of merged spots. Following this procedure, spot intensities are obtained by integration of the Gaussian function. The mathematical description of the spots is used both for data reduction and for increasing evaluation speed, since reevaluation of data after an image change takes only fractions of a second.

The hit rate of automatic spot detection is highly dependent on the quality of the 2-D gels. Correction capabilities of PDQuest software can be used to add undetected spots to the list of spots or to delete spots that arise from gel artifacts.

Gel Comparison

The next step in 2-D gel evaluation is the identification of proteins that are present in all gels of a series. This task is made difficult primarily because of inherent irreproducibility in gels, which affects the positions of spots within a gel series. Gel analysis software must detect minor shifts in individual spot position within the gel series. Many software packages for automatic gel comparison are created with the assumption that the relative positions of spots are altered only slightly relative to each other, and allocate the spots on this basis. Prior to automatic gel comparison, PDQuest software selects the best 2-D gel of a gel series as a reference or standard gel and compares all other 2-D gels to this gel. Proteins in a gel series that are not present in the reference gel are added manually so that the reference gel will include all proteins of a gel series.

Before the software can detect and document matching of different spots, a number of landmarks, or identical spots in the gel series, must be manually identified. The landmarking tool speeds the process by making "best guess" assignments of landmark spots to images in the gel series. With PDQuest software, it is possible to simultaneously display up to 100 enlarged details of 2-D gels on the screen. This simultaneous display of all 2-D gels of a test series enables rapid and error-free determination of the fixed points.

Using the landmarks, the image analysis software first attempts to compare all spots lying very near these fixed points and then uses the matched spots as starting points for further comparisons. Thus, the entire gel surface is systematically investigated for the presence or absence of matching spots in a gel series. The results of the automatic gel comparison require verification, as does automatic spot detection. Two tools assist this verification process in PDQuest: Either identical protein spots are labeled with matching letters and allocated section by section, or the deviations in the spot positions of a particular 2-D gel can be displayed as lines that show spot shifts in comparison to the reference gel.

Data Analysis

With PDQuest software, all gels of an experiment are viewed as a unit. To compare gels from different experiments, the reference images are compared. In such comparisons, each spot is automatically assigned a number so that identical spots have identical numbers. Experimental data can also be analyzed statistically — both parametric and nonparametric tests are available.

For ordering information related to this section see page 50

Chapter 8 — Identification and Characterization of 2-D Protein Spots

Sequence Data from 2-D Gels

2-D electrophoresis has the virtually unique capability of simultaneously displaying several hundred gene products. 2-D gels are an ideal starting point for protein chemical identification and characterization. Peptide mass fingerprint or sequence data can be derived following 2-D electrophoresis with mass spectrometry or amino acid sequence analysis (Eckerskorn et al. 1988, Ducret et al. 1996). The sensitivity of currently available instruments makes 2-D electrophoresis an efficient "preparative" analytical method. Most current protein identification depends on mass spectrometry of proteins excised from gels or blots.

Integration of Image Analysis With Automated Spot Cutting

Image analysis software obtains quantitative and qualitative information about the proteins in a sample, and stores the information in files, which may also contain additional annotations (Figure 8.1).

The ProteomeWorks™ spot cutter (Figure 8.2) expands the capabilities of proteome labs by integrating PDQuest™ image analysis software. The image analysis files acquired by PDQuest direct automated spot cutting. Excised protein spots are deposited into microtiter plates ready for further automated processing. PDQuest software tracks the protein spots through spot cutting and protein identification. Downstream protein spot identifications are generally obtained from peptide mass fingerprint analysis using mass spectrometry.

The ProteomeWorks spot cutter is a precision instrument with a small benchtop footprint. It is fully automatic to increase throughput and minimize the amount of hands-on time spent excising protein spots. The spot cutter individually excises even overlapping spots for unique identification.

Automated Protein Digestion

The ProteomeWorks spot cutter eliminates the first of two bottlenecks for excision and enzymatic digestion of protein spots. Driven by PDQuest software, it enables automated spot excision and deposition of cut gel spots into microtiter plate wells. Isolated proteins from the gel pieces are then digested to release peptides for detailed sequence analysis by mass spectrometry, leading to protein identification.

Excised gel spots can be robotically destained, chemically modified (reduced and alkylated), and digested in preparation for either MALDI-TOF-MS or ESI-MS-MS mass spectrometry with the Micromass Mass*PREP* station. Each process is executed under fully automated software control with a range of standard protocols enabling high throughput and flexibility.

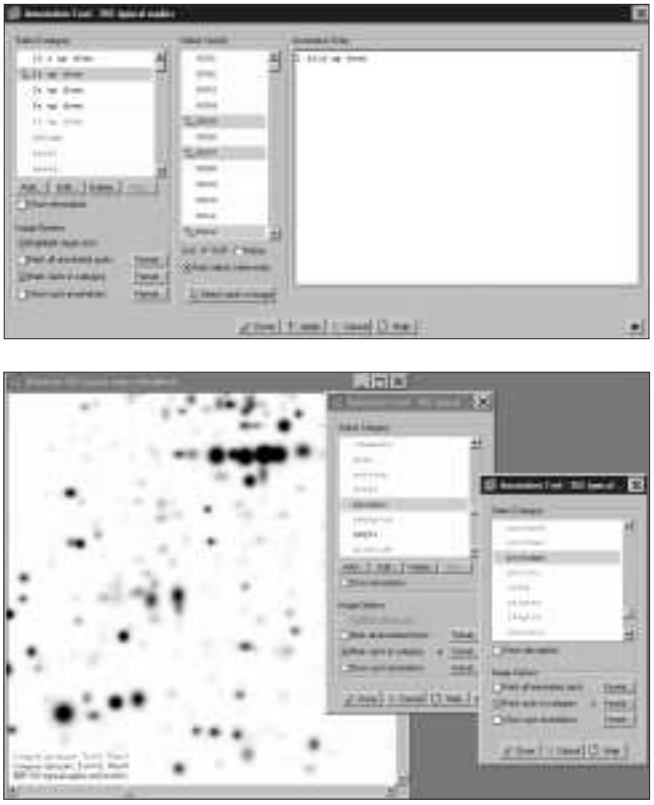

Fig. 8.1. PDQuest software's increased flexibility includes simultaneous display of multiple annotation categories.

Fig. 8.2. The ProteomeWorks spot cutter.

Manual protein digestion is a tedious, time-consuming process that is subject to variability and keratin contamination. Automation of this process with the Mass*PREP* station eliminates a significant bottleneck for high-throughput protein identification.

For ordering information related to this section see page 50

Operational features of the Mass*PREP* station include a variable temperature control for optimized reduction, alkylation, and digestion of proteins, and onboard cooling capabilities for reagents and peptide digests to ensure reproducible digestion results. The station employs a variety of sample cleaning technologies (Mass*PREP* targets and Millipore ZipTip pipet tips) to prepare peptide digests prior to automated deposition of samples onto a M@LDI or Mass*PREP* target plate. Contamination of peptide samples is also minimized with the Mass*PREP* clean air enclosure. For more information visit the Micromass web site at **www.micromass.co.uk**

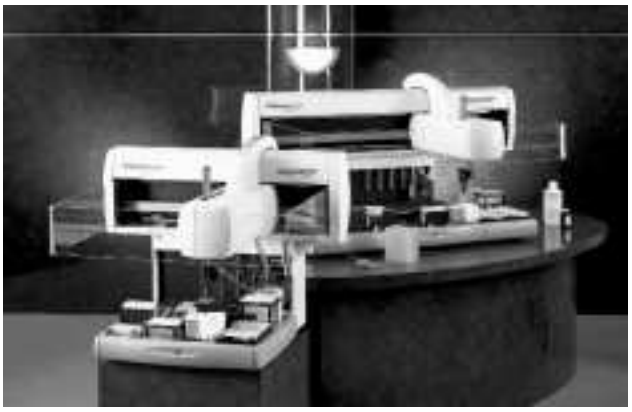

Fig. 8.3. Mass*PREP* station: the in-gel digestion and sample preparation robot of the ProteomeWorks system.

Rapid, High-Throughput Protein Identification by MALDI-TOF-MS

Peptide mass fingerprinting of protein digest products using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) provides an ideal method for protein identification when samples have been separated by 2-D PAGE. The M@LDI HT is one of a new generation of networked "2-D gel-MS" analyzers for high-throughput protein identification. M@LDI HT is the primary MS data acquisition device of the ProteomeWorks system, and features a fully automated target plate auto-changer for increased throughput. Networking enables distribution of data capture, protein assignment, and result presentation functions of ProteinLynx Global *SERVER* software within a secure client-server architecture, maximizing computing power to quickly identify proteins.

Fig. 8.4. M@LDI HT, the "2-D gel-MS" analyzer of the ProteomeWorks System. Q-Tof *micro* with capillary HPLC optimized for high-sensitivity LC-MS-MS in post-genomic studies.

The M@LDI HT enables automated acquisition of optimized mass spectra and the derivation of monoisotopic peptide mass fingerprint information. Interrogation of multiple FASTA databases using Global *SERVER* software following capture of MS results provides rapid identification of proteins that fit the samples' peptide mass fingerprint, along with a confidence score indicating the validity of the identifications. Following MS identification, peptide mass fingerprint spectra and all of the identification results are available through electronic reports. In addition, protein identification results are seamlessly integrated with the gel image in PDQuest software. Figure 8.5 shows the data for a MS identification and how this information is accessible in PDQuest as an annotation to a specific gel spot by simply clicking on the spot.

Using this system, the working time to process data from spot cutting to protein digestion to MS analysis and image annotation is reduced by over 50% compared to manual processing of gel samples, with a corresponding reduction in error. All of the instrumentation and software in this process is part of the integrated ProteomeWorks system, a set of powerful tools for proteomic analysis.

Advanced Protein Characterization with ESI-LC-MS-MS and MALDI-MS-MS

MALDI-TOF MS provides an ideal high-throughput solution for protein identification; however, where protein identity is ambiguous, known databases must be searched with a higher degree of sequence information. The Micromass Q-Tof family of MS-MS instruments incorporates quadrupole/orthogonal acceleration time-of-flight (Q/oa-TOF) technology, enabling exact mass measurement and acquisition of the highest level peptide sequence information for de novo sequencing and BLAST analyses. The Q-Tof *Ultima* family of MS-MS instruments provides a flexible research platform for optimal results with either API LC-MS-MS or MALDI MS-MS, or with a combined platform for both API and MALDI MS-MS.

Protein digest samples in microtiter plates, prepared with the Mass*PREP* station, can be transferred directly to the Micromass CapLC (capillary HPLC) system for automated injection into the Q-Tof *micro* for integrated LC-MS-MS under MassLynx software control. The capability for MS to MS-MS switching "on the fly" with the Q-Tof family of instruments maximizes the amount of amino acid sequence information that can be generated with these instruments. MassSeq software also provides the capacity for automated de novo amino acid sequencing based on the MS results. For more information, contact **www.micromass.co.uk**

Fig. 8.5. The integration of ProteinLynx Global *SERVER*, the Micromass proteomics application manager and web-enabled database search engine, with PDQuest software enables annotation of gel images with protein identity and retrieval of MS data and database search results for all spots of interest.

Fig. 8.6. The Q-Tof *Ultima* family of electrospray LC-MS-MS systems provides the tools of choice for proteomics in the postgenomic era.

Part II

Methods

sample solubilization and preparation methods

first-dimension separation methods

second-dimension separation methods

methods for protein detection in gels

Chapter 9 — Sample Solubilization and Preparation Methods

Protein solubilization is sample dependent. Several solubilization solutions and protocols are detailed in this chapter. See pages 3–5 for background information on sample solubilization.

Standard Sample Solubilization Solution

The sample solubilization solution described in Table 9.1 is commonly used as a general extraction solution and provides a good starting point for sample preparation. This solution is also used for IPG strip rehydration, for sample application by in-gel rehydration or cup loading, and for sequential extraction of more complex samples. It is available from Bio-Rad as reagent 2 in the ReadyPrep™ sequential extraction kit (see page 28). A similar IPG rehydration/sample buffer ideal for *E. coli* samples can be ordered as ReadyPrep 2-D starter kit rehydration/sample buffer. Each vial in the starter kit reconstitutes to 10 ml of 8 M urea, 2% CHAPS, 40 mM DTT, 0.2% Bio-Lyte® 3/10 ampholyte.

Table 9.1. Sample solubilization solution (ReadyPrep reagent 2).

Components	Amount
8 M Urea	47 ml of 8.5 M stock (Table 9.2) or 24 g dry urea dissolved in 25 ml of H_2O
50 mM DTT or 2 mM TBP	385 mg or 500 µl of 200 mM TBP stock
4% CHAPS	2 g
0.2% Carrier ampholytes	See Table 9.4
0.0002% Bromophenol Blue*	100 µl of 0.1% stock
Water	Adjust to 50 ml

Dissolve the urea in about 25 ml of water with stirring. Add the CHAPS, ampholytes, and Bromophenol Blue and adjust the solution to a final volume of 50 ml. Tris may be added at 10–40 mM if pH control is important. Tris will increase the conductivity and extend the time required to focus the IPG strips. Add DTT or TBP immediately before use. Use carrier ampholytes that span the pH range of the IPG strip according to Table 9.4. Urea stock solutions should be used soon after they are made, or treated with a mixed-bed ion exchange resin to avoid protein carbamylation by cyanate, which forms in old urea. Table 9.2 and the section on urea stock solutions describe the preparation and storage of urea stock solutions. Store sample solubilization solution in aliquots at -20ºC. Thaw only the required number of aliquots and discard leftover solution. Add sample solution to the protein sample so the final concentration of urea is ≥6.5 M. Solid urea may be added as necessary. Proteins may be directly extracted in sample solubilization solution using at least 9 ml of solution for each 1 ml of packed cell pellet. Use sample solubilization solution to rehydrate IPG strips.

Table 9.2. Urea stock solution, 8.5 M.

Urea Stock Solution Components	Amount
8.5 M Urea	510 g
Water	Adjust to 1 L

Dissolve the urea in about 600 ml of water with gentle heating (<30°C) and vigorous stirring with a heavy stirbar. Remove from the heat source as soon as the urea dissolves. Add 5 g of Bio-Rad deionizing resin (Bio-Rex® 501-X8) and stir for 10 min. If the resin de-colors, add an additional 5 g and repeat until the resin no longer loses color. Filter the solution through Whatman No.1 paper using a Buchner funnel.

Store convenient aliquots of this urea solution at -20°C until required. This deionized 8.5 M stock can be used to make up all urea-containing solutions. Do not store urea solutions at room temperature (or 4°C) any longer than necessary. Urea in solution exists in equilibrium with ammonium cyanate, which can cause irreversible protein modification and interfere with IEF.

Urea Stock Solutions

Urea is a chaotropic agent comonly used in IEF sample preparation (see discussion on page 3). To prepare an 8.5 M urea stock solution, see Table 9.2. High-purity urea should be used for IEF. Charged species can be removed by addition of a mixed-bed ion exchange resin. The resin is then removed by filtration. Urea should not be heated above 30°C because carbamylation of the sample may occur, which gives rise to charged artifacts detected in the second-dimension gel.

For some applications, it is convenient to prepare a saturated urea solution (9.8 M) containing 4% CHAPS (Table 9.3). By diluting samples with the 9.8 M urea solution, the final urea and CHAPS concentrations remain high even when large volumes of aqueous protein sample must be denatured. The solution should be stored frozen in aliquots. Thaw enough for use when needed. Add reducing agent and ampholytes immediately before use. Discard unused reagent.

Table 9.3. Urea/CHAPS stock solution.

Components	Amount
9.8 M Urea	29.4 g
4% CHAPS	2 g
Water	Adjust to 50 ml

Dissolve the urea in about 25 ml of water with stirring. Add the CHAPS and adjust the final volume to 50 ml. Store in aliquots at -20°C.

* Bromophenol Blue is included in trace amounts in rehydration solutions both to view the rehydration of the strip with the solution and to observe the early stages of electrophoresis. It is not required for solubilization.

For background information related to this section see pages 3–7

Ampholytes for Sample Solutions

Ampholytes are added to all IPG rehydration buffers and sample solubilization solutions to help keep proteins soluble. The choice of ampholytes is dependent on the pH range of the IPG strip. Table 9.4 provides a starting point. See page 4 for further discussion.

Table 9.4. Suggested Bio-Lyte® ampholyte composition for IPG use.

IPG pH Range	Bio-Lyte Ampholyte (Stock) Range	Conc. (w/v)	Sample Solution Volume per 5 ml	per 50 ml
3–10	3/10	40%	25 µl	250 µl
4–7	4/6	40%	12.5 µl	125 µl
	5/7	40%	12.5 µl	125 µl
3–6	3/5	20%	25 µl	250 µl
	4/6	40%	12.5 µl	125 µl
5–8	5/8	40%	25 µl	250 µl
7–10	7/9	40%	12.5 µl	125 µl
	8/10	20%	25 µl	250 µl

Enhanced Solubilizing Solutions

Recent improvements to protein solubilization include addition of multiple chaotropic agents, multiple surfactants, or both to the solubilizing solution. For some samples, solutions that have strong chaotropic properties extract more proteins than the standard sample solution (see page 3). In other instances, proteins are better solubilized by multiple surfactants (see page 4). Each protein sample is different, and the most efficient solubilizing solution can only be determined by trial.

Multiple Chaotropic Agent Solution

This solution can be made according to the directions in Table 9.5. Tris may be added at 10–40 mM if pH control is important for a particular sample. Tris will increase the conductivity and extend the time required to focus the IPG strips. The reducing agent TBP should be added immediately prior to use.

Table 9.5. Multiple chaotropic agent solution preparation.

Components	Amount
7 M Urea	2.1 g dry urea and 3 ml of water to dissolve, or 4.1 ml of 8.5 M stock as prepared in Table 9.2
2 M Thiourea	760 mg
2 mM TBP	50 µl of 200 mM TBP stock
4% CHAPS	200 mg
0.2% Carrier ampholytes	See Table 9.4
0.0002% Bromophenol Blue*	10 µl of 0.1% stock
Water	Adjust to 5 ml

* Bromophenol Blue is included in trace amounts in rehydration solutions both to view the rehydration of the strip with the solution and to observe the early stages of electrophoresis. It is not required for solubilization.

Multiple Surfactant Solution

Reagent 3 in the Bio-Rad ReadyPrep sequential extraction kit is a multiple surfactant-containing solution and can be purchased from Bio-Rad (see Table 2.1). Alternatively, it can be made according to the directions in Table 9.6.

The Tris in this solution inactivates some proteases. It may be omitted, in which case the conductivity will be reduced and the focusing time will be shortened. The reducing agent TBP should be added immediately prior to use.

Table 9.6. Multiple surfactant solution (ReadyPrep reagent 3).

Components	Amount
5 M Urea	1.5 g dry urea and 3 ml of water to dissolve, or 2.9 ml of 8.5 M stock as prepared in Table 9.2
2 M Thiourea	760 mg
2 mM TBP	50 µl of 200 mM TBP stock
2% CHAPS	100 mg
2% SB 3-10	100 mg
0.2% Carrier ampholytes	See Table 9.4
40 mM Tris	24.2 mg
0.0002% Bromophenol Blue	10 µl of 0.1% stock
Water	Adjust to 5 ml

Sequential Extraction of Proteins

See page 6 for background information and a flowchart (Figure 2.4) related to sequential extraction.

Sequential Extraction Protocol

Sequential extraction reagents are available premade in Bio-Rad's ReadyPrep sequential extraction kit. Alternatively, the composition of reagents is described in Table 9.7. The goal of the first step in sequential extraction is to lyse the cells of interest directly in sequential extraction reagent 1 using a physical lysis procedure. The method of lysis will vary depending on cell type. Follow standard procedures for cell or tissue growth and harvesting, and for physical cell lysis. For bacteria, growth medium can be washed away with reagent 1 before cells are lysed by sonication or French press. Animal tissues can be homogenized or sonicated in reagent 1. Plant tissues can be ground in liquid nitrogen and the resulting powder suspended in reagent 1. The 2-D results can be improved greatly by a nuclease treatment at this step. Thorough lysis is important for the best segregation of the different solubility classes of proteins. It may be necessary to separate organelles or to ensure their lysis; these approaches prevent organelle lysis during subsequent solubilization steps from affecting the protein classes obtained.

For ordering information related to this section see page 45

Table 9.7. Sequential extraction solutions.

Composition	Amount
Reagent 1 40 mM Tris	96.8 mg Tris in 20 ml water
Reagent 2 8 M urea, 4% CHAPS, 2 mM TBP, 40 mM Tris, 0.2% ampholytes	See Table 9.1, Sample solubilization solution
Reagent 3 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10, 2 mM TBP, 40 mM Tris, 0.2% ampholytes	See Table 9.6, Multiple surfactant solution

Extraction 1

1. Place the appropriate amount of sample to yield 50 mg of protein for each 1 ml of solution 1 into a suitable lysis vessel. The amount of material needed will vary according to the type of sample and must be determined empirically.
2. Lyse the cells according to standard protocols.*
3. Centrifuge to yield a firm pellet. A microcentrifuge spin at 12,000 x g for 8 min at room temperature is usually sufficient.
4. Collect the supernatant, label it as extract 1, and reserve the pellet for further extraction (in step 8). Determine the concentration of the supernatant as described on page 30.
5. Freeze the supernatant until ready for use in aliquots appropriate for a typical experiment (in duplicate).
6. When ready to load IPG strips, dilute the supernatant in reagent 2 (made according to step 7 below). Use an appropriate volume for the passive rehydration of IPG strips in the sample.

Extraction 2

7. Prepare reagent 2 by adding 10 µl of 200 mM TBP to each 1 ml of reagent 2 from the ReadyPrep sequential extraction kit, or prepare the sample solubilization solution described on page 27.
8. Wash the pellet from step 4 twice in reagent 1: vortex and centrifuge, then discard each supernatant. Add 1/2 volume of reagent 2 (compared to the volume of reagent 1 used in step 1) to the washed pellet. (To track extraction efficiencies and protein yields, determine the protein concentration of all washes before discarding them.)
9. Vortex the mixture for 5 min; some samples may also require sonication, or aspiration through a fine-gauge needle.*
10. Centrifuge the mixture to yield a firm pellet and a clear supernatant. A microcentrifuge spin at 12,000 x g for 8 min at room temperature is usually sufficient.
11. Collect the supernatant, label it as extract 2, and determine its protein concentration. Reserve the pellet for further extraction (in step 13). Freeze the supernatant until ready for use in aliquots appropriate for a typical experiment (in duplicate).

Extraction 3

12. Prepare reagent 3 by adding 10 µl of TBP per 1 ml of reagent 3 from the ReadyPrep extraction kit or as the multiple surfactant solution described on page 26.
13. Optional step: Wash the pellet twice with reagent 2 and discard the supernatants.
14. Add the same volume of reagent 3 to the pellet from step 13 as the volume of solution 2 added in step 8.
15. Vortex and centrifuge as described for the second extraction.
16. Centrifuge and recover the supernatant and label it as extract 3. Determine the protein concentration.
17. Freeze the supernatant until ready for use in aliquots appropriate for a typical experiment (in duplicate).
18. The pellet can be further extracted with SDS to dissolve highly insoluble proteins for protein analysis in 1-D SDS-PAGE.

Endonuclease Treatment

Add 150–300 U of endonuclease to each 1 ml of sample dissolved in the selected solubilization solution. Incubate the sample at room temperature for at least 20 min. The amounts and times necessary will vary depending on the sample.

For a discussion on the removal of nucleic acids, see page 7.

* Optional: Add 150 U of endonuclease and mix. Incubate for 20 min at room temperature.

For background information related to this section see page 6

Protein Determination for IEF Samples

Protein assays are generally sensitive to detergents or reducing agents used at the concentrations found in typical sample solutions. The two assays described below are adaptations to the Bradford (modified Bio-Rad protein assay) or Lowry (*RC DC*™ protein assay) protein assays that compensate for the interfering substances. The modified Bio-Rad protein assay procedure is simple to perform, but the mechanism for this protein assay, dye binding, will give variable results with different proteins. However, it will give consistent results within a protein mixture, and is useful to standardize protein loads. The *RC DC* protein assay involves more steps, but is less variable for different proteins because the reaction is with the peptide bond, not the amino acid side chains.

The SmartSpec™ 3000 spectrophotometer is a scanning tabletop UV/visible spectrophotometer that measures absorbance from 200 nm to 800 nm. It is ideal for quantitation of the results of either the Bradford or *RC DC* protein assays.

Modified Bio-Rad Protein Assay

This modification to the standard Bio-Rad protein assay (Figure 9.1) is recommended to determine the protein content in typical sample solutions used to load IPG strips.

Materials:

- Bio-Rad protein assay kit I: contains 450 ml dye reagent concentrate and a bovine γ-globulin standard
- Deionized water
- Whatman No. 1 filter paper or equivalent (easiest to use if purchased fluted for funnel filtration)

  Concentrated HCl to make a 0.12 N stock
- 20 µl of each sample (40 µl for duplicates)
- IPG sample buffer, protein-free

Procedure:

1. Prepare the bovine γ-globulin standard at 14 mg/ml by reconstituting the lyophilized protein in 1 ml of water. This is 10x the concentration that is recommended in the kit instructions.
2. Prepare a 1:4 dilution of the dye reagent concentrate by mixing 1 part of dye with 3 parts of water, and filter the dye through Whatman No. 1 filter paper. (Each assay point requires 3.5 ml of diluted dye reagent.)
3. Prepare 0.12 N HCl (nominal) by diluting concentrated HCl 1:100.
4. Prepare a standard curve covering the range of 0.1–14 µg protein/µl by diluting the 14 mg/ml standard in the same buffer as the sample.
5. Mix 20 µl of each standard or sample with 80 µl of 0.12 M HCl in separate assay tubes. It is a good idea to make duplicates for each sample or standard.
6. Add 3.5 ml of diluted dye reagent to each tube. Vortex gently.
7. After 5 min, measure the absorbance of each sample or standard at 595 nm.
8. Plot the absorbance values versus the amount of protein (in µg) for the standard curve. Expect a nonlinear relationship.
9. Compare the absorbance readings for the samples to the standard curve.

Fig. 9.1. The Bio-Rad protein assay can be performed in tubes, microtubes, or microtiter plates.

For ordering information related to this section see page 45

Table 9.8. Reagent compatibility with the *RC DC* protein assay.

Compatible 2-D Sample Buffer Components	After 1 Wash	After 2 Washes (Optional)
Dithiothreitol (DTT)	100 mM	350 mM
Tributylphosphine (TBP)	2 mM	-
β-Mercaptoethanol	5%	10%
ReadyPrep sequential extraction reagent 2*	Not compatible	Full strength†
ReadyPrep sequential extraction reagent 3**	Not compatible	Full strength†
Laemmli buffer (with 5% β-mercaptoethanol)	Full strength	-
CHAPS	2%	-
Tween 20	2%	-
Triton X-100	2%	-

* Contains 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 0.2% (w/v) Bio-Lyte 3/10 ampholyte, 2 mM TBP
** Contains 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 40 mM Tris, 0.2% (w/v) Bio-Lyte 3/10 ampholyte, 2 mM TBP
† The presence of these substances changes the response of protein to the assay reagents. Protein standards should be prepared in the same buffer as the protein samples.

RC DC Protein Assay

The 2-D sample buffer components listed in Table 9.8 are compatible with the *RC DC* protein assay. The presence of one or more of these substances may change the response of the protein to the assay reagents. Thus, the protein standard should always be prepared in the same buffer as the protein sample (see Figure 9.2).

Materials:

- Bio-Rad *RC DC* protein assay kit 1, contains *RC* reagents package (*RC* reagent I, 250 ml; *RC* reagent II, 250 ml), *DC*™ protein assay components (*DC* reagent A, 250 ml; *DC* reagent B, 2 L; *DC* reagent S, 5 ml), and bovine γ-globulin standard sufficient for 500 assays
- Deionized water
- 25 µl of each sample (50 µl for duplicates)
- IPG sample buffer

Microcentrifuge Tube Assay Protocol (1.5 ml)

1. Add 5 µl of *DC* reagent S to each 250 µl of *DC* reagent A that is needed for the assay. This solution is referred to as reagent A´. Each standard or sample assayed will require 127 µl of reagent A´.

   (Reagent A´ is stable for 1 week even though a precipitate may form after 1 day. If a precipitate forms, warm the solution and vortex it. Do not pipet the undissolved precipitate as it will likely plug the tip of the pipet and change the volume of reagent A´ added to the sample.

2. Prepare 3–5 dilutions of a protein standard ranging from 0.2–1.5 mg/ml protein. A standard curve should be prepared each time the assay is performed.

   (For best results, the standards should always be prepared in the same buffer as the sample.)

3. Pipet 25 µl of each standard and sample into a clean, dry microcentrifuge tube.
4. Add 125 µl *RC* reagent I into each tube and vortex. Incubate the tubes for 1 min at room temperature.
5. Add 125 µl *RC* reagent II into each tube and vortex. Centrifuge the tubes at 15,000 x g for 3–5 min.
6. Discard the supernatant by aspiration, then invert the tubes on clean absorbent tissue paper. Allow the liquid to drain completely from the tubes.
7. Add 127 µl of reagent A´ to each microcentrifuge tube and vortex. Incubate the tubes at room temperature for 5 min or until the precipitate is completely dissolved. Vortex before proceeding to the next step.
8. Add 1 ml of *DC* reagent B to each tube and vortex immediately. Incubate at room temperature for 15 min.
9. After the 15 min incubation, read the absorbance at 750 nm. The absorbance should be read within 1 hr.

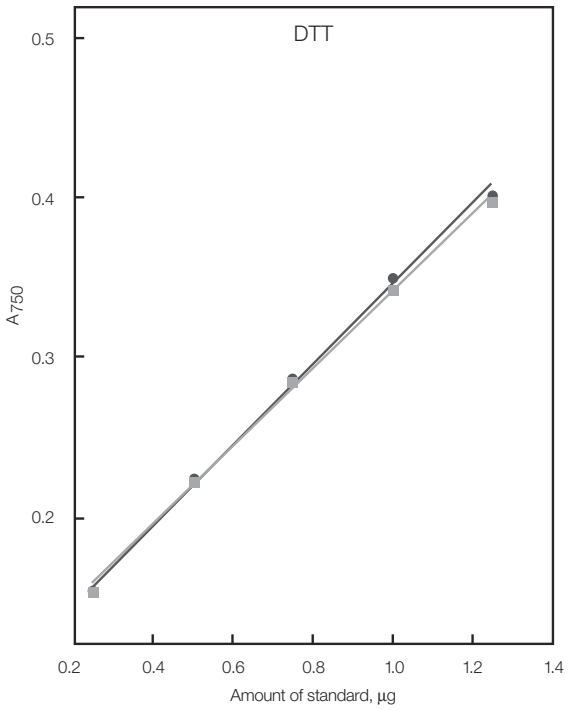

Fig. 9.2. Effect of reducing agent on standard curve. Gray line, carbonic anhydrase standards without reducing agent; black line, with 350 mM DTT.

For ordering information related to this section see page 45

Chapter 10 — First-Dimension Separation Methods

Master 2-D techniques before proceeding to separate your own samples with the ReadyPrep™ 2-D starter kit. Premixed reagents, a standardized sample, and a detailed optimized protocol allow you to get familiar with 2-D techniques and to validate your 2-D system. See page 45 for ordering information.

Protein Load for 2-D Gels

Table 10.1 shows generally recommended protein loads for 2-D gels. Because of sample-to-sample variation, the amounts are a guide only. For narrower pH range IPG strips, more protein can be loaded, because proteins outside the range of pI resolution will not remain on the strip to enter the 2-D gel. For single-pH-unit IPG strips, the amount that can be loaded can be as much as 4–5 times more, which allows better detection of low-abundance proteins. For further discussion of factors related to protein load, see page 7.

Table 10.1. Approximate protein loads for IPG strips.

IPG Strip Length	Analytical Load (Silver or SYPRO Ruby staining)	Preparative Load (Coomassie staining)
7 cm	10–100 µg protein	200–500 µg protein
11 cm	50–200 µg protein	250–1,000 µg protein
17 cm	100–300 µg protein	1–3 mg protein

IPG Strip Rehydration

For a discussion of IPG rehydration and sample application, see pages 10–11.

Solutions used to rehydrate IPG strips prior to loading a sample are the same as those used to solubilize or dilute samples for in-gel rehydration (see page 27). Methods for rehydration of strips in buffers (with or without sample) are described in the following sections.

Passive Rehydration With Sample

Passive sample application during rehydration is performed by placing the IPG strip gel side down in the channel of a focusing or rehydration tray that contains the sample in an appropriate rehydration solution. Use the sample volumes given in Table 10.2. This procedure will result in rehydration of the strips to their original thickness of 0.5 mm. Larger or smaller volumes can be used and the strips will swell to accommodate more liquid up to a point (Görg et al. 2000). A minimum of 11 hr total rehydration time is recommended. It is important that the strips be left in the well for the entire time, even if it appears that all of the liquid has been absorbed. High MW proteins cannot enter the gel until the pores are large enough to accept them, which only occurs when the pores have swelled to their maximum size.

Table 10.2. Approximate volumes to hydrate ReadyStrip™ IPG strips.

ReadyStrip IPG Strip Length	Volume
7 cm	125 µl
11 cm	185 µl
17 cm	300 µl

If too much solution remains outside the gel in the focusing tray during electrophoresis, a parallel current path along the surface of the strip can form in which the proteins will not be focused. This can result in protein loss and streaking. To minimize the possibility of a parallel current path, rehydrate the strips in a disposable rehydration tray, then transfer them to the focusing tray. During transfer, carefully blot excess liquid from the strip with moist filter paper prior to beginning the run.

Remove the IPG strip from the protective cover using gloved hands and forceps. Carefully place the IPG strip in the rehydration buffer, gel side down, making sure the entire strip is wetted. There is no “best way” to place dry IPG strips in contact with solution in the trays. Any of the methods illustrated in Figure 10.1 are suitable.

It is helpful to add a trace of Bromophenol Blue to the sample solution to observe the hydration process. Allow the liquid to distribute for about 1 hr before covering the strips with mineral oil. The IPG strips must be covered to prevent evaporation, which will cause the urea to precipitate as it becomes more concentrated. As a precaution against evaporation, mineral oil should be gently layered on top of each channel until it completely covers each strip.

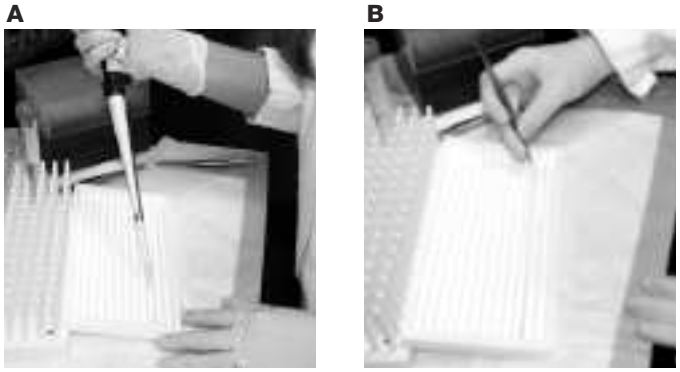

Fig. 10.1 A and B. Strip rehydration method 1.
Prop up one of the long edges of the tray at an angle to the lab bench. Pipet the rehydration solution along the entire length of the lower corner of each channel (A); place the strip, edge first, into the liquid (B). Then place the tray flat on the benchtop.

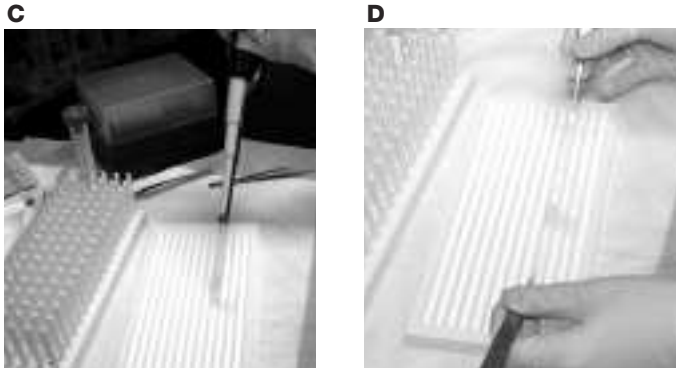

Fig. 10.1 C and D. Strip rehydration method 2.
Pipet the rehydration solution into the middle of each tray channel (C); bend the strip into a “U” shape and lower it into the liquid from the center out to the edges (D).

For background information related to this section see pages 8–12

E

Fig. 10.1 E. Strip rehydration method 3.
Pipet the rehydration solution into one end of each tray channel. Butt the strip up to the same end of the channel and lower it into the liquid toward the opposite end (E).

Active Rehydration

For active rehydration of IPG strips with sample in a focusing tray, run the IEF cell under low voltage (50 V). Ensure that the liquid extends past the electrode wires at each end so that the entire strip rehydrates and no dry area creates a discontinuity in the current path. It might be necessary to lift the ends of the IPG strip slightly to get the liquid to flow to the ends of the strip. After the sample has been in contact with the strips for 1 hr, add mineral oil to cover each strip. The PROTEAN® IEF cell can be programmed for active rehydration and to transition automatically into a focusing run. Alternatively, a pause may be incorporated to allow the operator to insert a wick under each end of the strip (see the section below on performing IEF).

If this method of sample application causes a disproportionate ratio of large proteins to small proteins, try passive rehydration.

Performing IEF

The PROTEAN IEF cell with integrated power supply and Peltier cooling is recommended for IEF protocols in this manual. It can simultaneously run up to twelve 11 or 17 cm IPG strips or up to twenty-four 7 cm strips. Running conditions can be better controlled by running the same type of sample, buffer, and IPG strip pH range together. See Chapter 3 for a general discussion of IEF and pages 11–12 for a discussion of running conditions that affect results.

Positioning Strips and Use of Wicks

After the strips have rehydrated, move them to the IEF focusing tray if they were rehydrated in other trays. Carefully blot excess liquid from the strip with moist filter paper.

Wicks are highly recommended because they collect salts and other contaminants in the sample. Without wicks, salts collect at the anode and cathode, producing high conductivity that can alter the gradient, cause discontinuities in the gel, and cause “hot spots” or burns. Place a dry wick on each electrode that is used (Figure 10.2). Position the wicks within the indentations of the channels. Pipet 5–8 µl of water on each wick before positioning the IPG strips.

Fig. 10.2. Placement of wicks on the electrodes in each channel that will be used. IPG strips will be placed on top of the wicks.

Alternatively, if strips are rehydrated in the focusing trays (either actively or passively), the ends of each strip can be lifted with forceps and wet wicks inserted between the strip and the electrodes (Figure 10.3). Wicks should be wetted but not soaked. Blot wetted wicks before placing them in the tray.

Fig. 10.3. Insertion of wicks under both ends of an IPG strip that has been rehydrated in a focusing tray.

Cover the strip with mineral oil before starting the focusing run to prevent evaporation and carbon dioxide absorption during focusing. Channels should be filled nearly to the top but should not be overflowing. The PROTEAN IEF focusing tray has rounded corners at both ends of the individual channels that prevent mineral oil movement into the adjacent channels. The rounded corners also reduce salt buildup due to inadequate cleaning between IEF runs. It is important to clean the focusing trays properly between runs. Channel-to-channel leakage is common when salts accumulate in the channels.

For ordering information related to this section see pages 45–46

Focusing Conditions for IPG Strips on the PROTEAN IEF Cell
See pages 11–12 for further discussion of running conditions for IPG strip focusing. Table 10.3 gives suggested total volt-hours for IPG strip runs. These conditions are intended as a guide; individual samples may require more or less time.

Table 10.3. Suggested focusing conditions.

ReadyStrip™ IPG Strip Length	Maximum Voltage	Typical Volt-Hours
7 cm	4,000 V	7,000–10,000 V-hr
11 cm	8,000 V	20,000–40,000 V-hr
17 cm	10,000 V	30,000–60,000 V-hr

Voltage Ramping Modes
Voltage ramping can replace traditional stepwise voltage programming with continuous voltage changes. The PROTEAN IEF cell (Figure 10.4) includes three voltage ramping modes: rapid, linear, and slow. Each ramping mode is appropriate for the resistance of particular samples. The combined resistance of the IPG strips, the rehydration buffer, and the sample determines which ramping mode should be used. During the focusing process, charged contaminants move to the electrodes and proteins move to the pH equal to their pI. While the proteins are being focused, the resistance of the IPG strip gradually increases until it reaches a maximum.

Each voltage ramping mode controls the rate of voltage change as follows:

Rapid ramping mode — In rapid ramping mode, salts and other ionic contaminants are driven from the IPG strips as rapidly as possible. The limiting factor in reaching the maximum set voltage is the current limit per strip. The maximum voltage can be reached in ≤2 hr for high-resistance (low-ionic-strength) samples, or in >6 hr for low-resistance samples. In both cases the power supply will run at the set current limit until a steady state is reached. This is the mode of choice for many samples, and is particularly useful to minimize low-resistance sample run time.

Linear ramping mode — In linear ramping mode, the voltage increases linearly within the programmed time frame, starting with the final voltage of the previous step and ending with the maximum voltage programmed. The resistance of the sample/rehydration buffer system will determine whether the maximum set voltage can be reached in the programmed time. This mode is used for samples of intermediate resistance.

Slow ramping mode — In this mode, the voltage is increased quadratically:
$V = B + (N^2 \times (E - B)/T^2)$

where B = starting voltage, E = ending voltage, N = elapsed time, and T = total time. The run will continue below or at the current limit. This mode is used for high-resistance sample/rehydration buffer systems to minimize high power input initially while achieving high voltage as quickly as possible.

NOTE: The default current limit in the PROTEAN IEF cell is 50 µA per strip. A higher current limit, up to 99 µA per strip, can be programmed into a method. All preset methods have a fixed current limit of 50 µA per strip. In the rapid ramping mode, the system runs at the set current limit and adjusts the voltage until the maximum voltage is reached. In the linear or slow ramping modes, the system follows a specific algorithm and does not always run at the current limit. The factor that determines the time needed to reach maximum voltage is the composition of the sample solution. Systems with high salt concentration and high sample loads require a long time to reach steady state. It is not always possible to reach the maximum set voltage within the programmed time. High ampholyte concentrations and high protein load also limit the final attainable voltage.

Storage of IPG Strips After IEF

Because the pH gradient is fixed in the IPG strip gel, focused proteins are more stable at their pI than in conventional IEF gels. Focused IPG strips can be stored at -20°C indefinitely without affecting the final 2-D pattern. IPG strips are bound to a plastic sheet, so gel cracking, which results from expansion and contraction during freezing and thawing, is avoided and the IPG strips retain their original dimensions after thawing. It is convenient to store IPG strips in rehydration trays or screwcap plastic tubes, which can then be used to equilibrate the strips for the second dimension (see page 36).

Fig. 10.4. The PROTEAN IEF cell and accessories.

For background information related to this section see pages 11–12

Chapter 11 — Second-Dimension Separation Methods

Using Precast Gels

For discussion of precast gels and for a full list of the three sizes of Bio-Rad precast gels with IPG wells, see page 14.

To embed IPG strips onto precast gels, remove the gels from their protective wrapping and remove any tape that seals the bottom of the gels. Follow the instructions for IPG equilibration and agarose embedding (page 36).

Casting SDS-PAGE Gels Using Multi-Casting Chambers

In general, proteomics work requires running several IPG strips and second-dimension gels per experiment. It is important that gels have a very similar composition. The best way to ensure that handcast gels have the same composition is to cast them at the same time in a multi-casting chamber (Figure 11.1). This is especially important when casting gradient gels. Details of the assembly and use of multi-casting chambers are available in their accompanying instruction manuals. Tips that generally apply to all multi-casting systems are:

- Before assembling the casting chamber, glass plates should be carefully cleaned with Bio-Rad cleaning concentrate and thoroughly rinsed with deionized water
- Each pair of glass plates (two per gel) should be separated from the next by a spacer sheet; the spacer sheet allows easier separation of the cassettes after gel polymerization
- The volume of gel solution should be determined by measuring the volume of water needed to fill the assembled glass plates to the desired level in the multi-casting stand
- Allow overnight polymerization to compensate for the low concentrations of catalysts (recommended to ensure that polymerization does not start while the gradient gels are being cast)

Running Multiple Gels

Bio-Rad's Dodeca™ cells allow electrophoresis of 12 gels simultaneously under identical conditions, providing high throughput and reproducible results. Three size formats are available (see pages 14–15, Figure 4.2, and Table 4.2). Refer to the instruction manuals for specific directions on assembly and use.

Fig. 11.1. Apparatus for casting multiple gels. Multi-casting chambers for 12 PROTEAN Plus™ 3 gels or for 12 Mini-PROTEAN® gels allow uniform casting of gradient gels. Gradient makers are available for both size formats.

For ordering information related to this section see pages 46–48

IPG Equilibration for the Second Dimension

To solubilize focused proteins and to allow SDS binding in preparation for the second dimension, it is necessary to equilibrate focused IPG strips in SDS-containing buffers. This step is analogous to boiling samples in SDS buffer prior to 1-D SDS-PAGE. See page 14 for further discussion of equilibration. Rehydration/equilibration trays sized for each size strip can be used for equilibration.

Equilibration protocol:

Place one strip, gel side up, in each channel and fill the channels successively with the equilibration buffers derived from the base buffer (Table 11.1). First incubate with gentle agitation in DTT equilibration buffer 1 for 10 min, then decant. Refill the channels with iodoacetamide equilibration buffer 2 and incubate again for 10 min. This method requires 2.5 ml of each solution per strip for 7 cm strips, 4 ml for 11 cm strips, and 6 ml for 17 cm strips. After equilibration, remove the IPG strip and embed it onto the prepared second-dimension gel as described in the following section.

Table 11.1. Equilibration base buffer.*

Reagents	Amount (Final Concentration)
Urea	36 g urea (6 M)
20% SDS	10 ml (2%)
1.5 M Tris/HCl, pH 8.8 gel buffer	3.3 ml (0.05 M)
50% Glycerol	40 ml (20%)
Water	Adjust to 100 ml

* This buffer may be frozen in aliquots. Lyophilized equilibration base buffer can be ordered as ReadyPrep™ equilibration buffer II, which reconstitutes to 20 ml.

DTT Equilibration Buffer 1

This buffer reduces sulfhydryl groups. To prepare it, add DTT to equilibration base buffer (Table 11.1) to 2% (200 mg/10 ml) immediately prior to use.

Iodoacetamide Equilibration Buffer 2

This buffer alkylates sulfhydryl groups. While the strip is incubating in equilibration buffer 1, add dry iodoacetamide to equilibration base buffer (Table 11.1) to 2.5% (250 mg/10 ml).

Placement and Agarose Embedding of IPG Strips

Position the second-dimension gel cassette so that it is leaning slightly backwards (approximately 30° from vertical). Place the IPG strip onto the long plate with the plastic backing against the plate. Slide the strip, face down, between the plates using a spatula to push against the plastic backing. Be careful not to damage the gel with the spatula. Make sure the IPG strip is positioned directly on top of the second-dimension gel. To secure the strip in place, overlay it with 0.5–1.0% molten agarose prepared in SDS-PAGE running buffer (a small amount of Bromophenol Blue can be added to the agarose overlay in order to track the ion front during the run). Use warm molten agarose; hot agarose may accelerate decomposition of the urea in the equilibration buffer. Bubbles may form under or behind the strip when adding the agarose overlay. These bubbles may disturb protein migration and must be removed. Immediately after overlaying, use the spatula to dislodge bubbles by tapping the plastic backing on top of the strip. Stand the gel upright and allow the agarose to set prior to loading the gel into the electrophoresis cell (see Figure 11.2).

A B

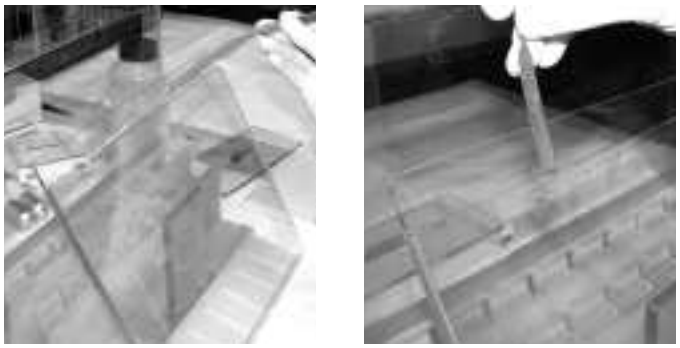

Fig. 11.2 A and B. In A, a 17 cm ReadyStrip™ IPG strip (held in forceps) is applied to the top of a PROTEAN XL Ready Gel® precast gel. The gel is held at an angle with the short plate forward. In B, the strip is pushed into direct contact with the top of the gel using a spatula.

C D

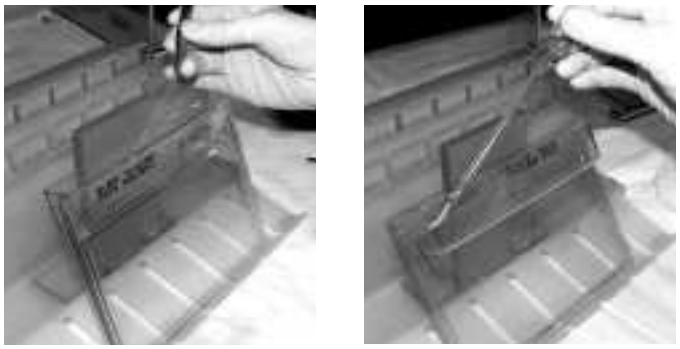

Fig. 11.2 C and D. In C, an 11 cm ReadyStrip IPG strip is applied to the top of a Criterion™ precast gel held at an angle. The strip is aligned so the plastic backing is against the back plate and the IPG strip is touching the top of the gel. Molten agarose may be added before the strip is placed in the well and the strip positioned within the liquid agarose, or the agarose may be added after the strip is in position (D). The gel is moved to an upright position while the agarose is setting.

For background information related to this section see pages 13–15

Running the Second Dimension

Second-dimension gels can be run in any cell appropriate to the size of the gel as shown in Table 4.2 (page 15). Bio-Rad Dodeca cells, which accommodate 12 gels per run, are well suited to high-throughput proteomics experiments. Figure 11.3 shows the use of precast gels in Dodeca cells.

When using PROTEAN Ready Gel precast gels with the PROTEAN Plus Dodeca cell, apply a thick bead of Vaseline petroleum jelly or other sealant between the glass plates along the downward facing edge of each cassette. The sealant prevents current leak and associated inward skewing of protein migration. PROTEAN Plus sandwiches are sealed along the downward facing edge and do not require sealant.

Insert the gels into the appropriate electrophoresis cell and run them according to the instruction manual provided with the cell.

A

B

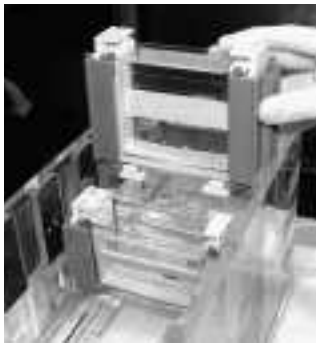

Fig. 11.3 A and B. In A, the seal is being removed from the bottom of a Ready Gel precast gel. Criterion precast gels and PROTEAN XL precast gels also must be unsealed. This can be done before the IPG strip is applied or after the agarose is set. In B, Ready Gel precast gels are being placed in the Mini-PROTEAN 3 Dodeca cell, which can run up to 12 gels at the same time.

C

Fig. 11.3 C. A gel is being inserted into the PROTEAN Plus Dodeca cell. The top of the gel is aligned with the agarose-embedded IPG strip at the cathode (negative electrode) side of the tank. This cell accommodates PROTEAN XL Ready Gel precast gels (shown), or PROTEAN Plus handcast gels up to 25 cm wide. A uniform electrical field is applied via metal plates. The pump visible behind the tank circulates the buffer to maintain a uniform temperature during the run.

Applying MW Standards

SDS-PAGE standards can be applied to gels that have no reference lane using this protocol:

1. Trim a PROTEAN IEF cell wick from 4 x 20 mm to 4 x 5 mm.
2. Pipet 10 µl of the SDS-PAGE standards onto the wick. Unstained standards can serve as a control for the staining procedure.
3. Slip the wick into the slot in the gel sandwich next to or overlapping an end of the IPG strip.
4. Seal the wick and the IPG strip with molten agarose.

For ordering information related to this section see pages 46–48

Chapter 12 — Methods for Protein Detection in Gels

Methods for dye staining, fluorescent staining, and silver staining of gels are provided in this chapter. The different protein detection methods are discussed on pages 16–17. Stain gels at room temperature with gentle agitation (e.g., on an orbital shaker). Use any convenient container that is appropriate to the method chosen. Silver staining should be done in glass, while SYPRO Ruby protein gel staining cannot be done in glass. Casserole dishes, photography trays, or any flat dishes large enough to allow the gels to lie flat are appropriate. Wear gloves and use good laboratory safety precautions when using staining chemicals.

Coomassie Brilliant Blue R-250 Stain

Use the following protocol for Coomassie Blue R-250 staining:

1. Stain the gel with gentle agitation in an ample volume of Coomassie Brilliant Blue R-250 staining solution (0.1% Coomassie Blue R-250 (w/v) in 40% methanol (v/v), 10% acetic acid (v/v)) for 20 min to 1 hr. This solution should not be reused.
2. Wash the gel in an ample volume of Coomassie Brilliant Blue R-250 destaining solution (40% methanol, 10% acetic acid). Lab tissues (e.g., Kimwipes) can be dropped in the destaining solution to help capture the dye and aid in destaining. Agitate gently, and change the solution until background staining has been removed.

Bio-Safe™ Colloidal Coomassie Blue G-250 Stain

Bio-Safe colloidal Coomassie stain (Figure 12.1) is a preformulated staining solution with sensitivity between that of Coomassie Blue R-250 and silver stains.

Use the following protocol for Bio-Safe Coomassie staining:

1. Rinse gels twice for 10 min in deionized water with agitation to remove SDS.
2. Add sufficient Bio-Safe Coomassie stain to cover the gels. Incubate with agitation for 1 hr to overnight. Color will continue to develop after 1 hr.
3. Rinse the gel with deionized water with agitation until desired contrast is achieved.

Fig. 12.1. Bio-Safe Coomassie stain is available in 1 L bottles or 5 L cubes.

SYPRO Ruby Protein Gel Stain

SYPRO Ruby protein gel staining is a simple procedure. Polymethylpentene dishes are ideal containers for staining because the high-density plastic adsorbs a minimal amount of the dye. Glass dishes are not recommended.

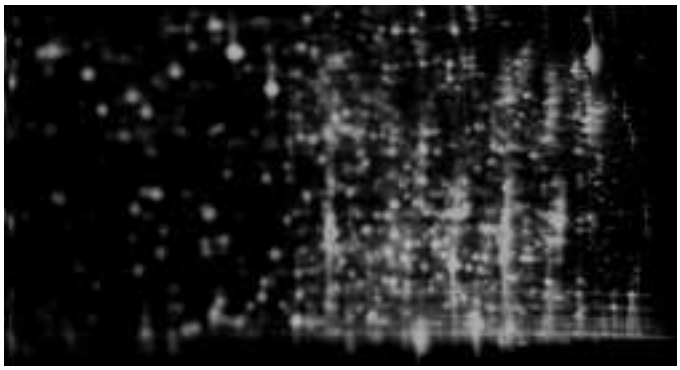

Fig. 12.2. A 2-D gel stained with SYPRO Ruby protein gel stain.

Follow this protocol to stain and visualize SYPRO Ruby-stained gels (Figure 12.2):

1. Fix gels for 30 min to 1 hr in a mixture of 10% methanol and 7% acetic acid.
2. Add SYPRO Ruby protein stain to the gel. Do not dilute the stain.

   (The minimum staining volumes for typical gel sizes are: 50 ml for 8 x 10 cm gels, 150 ml for 13.5 x 10 cm gels, 330 ml for 16 x 20 cm gels, and 500 ml for 20 x 20 cm gels; using too little stain will lower the sensitivity.)

   SYPRO Ruby protein gel stain is an end-point stain. Some staining can be seen in as little as 30 min. However, a minimum of 3 hr of staining is required for maximum sensitivity. For convenience, gels may be left in the dye solution overnight or longer without overstaining.
3. Prior to imaging the gel, to further decrease background fluorescence and to reduce speckling, the stained gel should be washed for at least 30 min in a mixture of 10% methanol and 7% acetic acid. The gel may be monitored by UV epi-illumination to determine the level of background fluorescence. Gels do not over-destain, although the fluorescent intensity of gels left in destaining solution or water for weeks will be reduced. Store stained gels in water.
4. Gels can be viewed on a “blue light” or UV box, or scanned on a Bio-Rad Molecular Imager FX™ Pro Plus or VersaDoc™ imaging system (see Chapter 7).

For background information related to this section see pages 16–17

Bio-Rad Silver Stain (Merril)

Silver staining is a highly sensitive method for detection of proteins and nucleic acids in polyacrylamide gels. The Bio-Rad silver stain, based on the method of Merril et al. (1981), is sensitive to 0.1 ng protein per mm^2 of gel, which is 10- to 50-fold more sensitive than Coomassie Brilliant Blue R-250 for proteins. This method is not recommended if mass spectrometry will be performed on spots excised from the gels. Use Silver Stain Plus™ stain, described in the following section, for mass spectrometry applications.

Follow the detailed instructions included in the kit. For large gels, the procedure involves 13 steps and may take up to 13 hr to perform.

Fig. 12.3. Bio-Rad Silver Stain Plus kit.

Silver Stain Plus Stain

Proteins can be visualized in 1 hr with very little hands-on time by using a carrier-complex silver-staining chemistry similar to that developed by Gottlieb and Chavko (1987) for detection of DNA in agarose gels. Silver Stain Plus stain is 30- to 50-fold more sensitive than Coomassie Blue R-250 dye and will detect nanogram amounts of protein. For optimal staining results, follow the instruction manual for the kit (Figure 12.3).

Gel Drying

The GelAir™ drying system is perfect for drying polyacrylamide gels up to 20 x 20 cm (Figure 12.4). After drying, the gels are completely clear and enclosed in cellophane. The dried gels are ideal for densitometry, photodocumentation, autoradiography, or long-term storage.

The GelAir dryer is a heated drying chamber that dries mini gels in 45 min or 20 x 20 cm gels in 60 min. The dryer holds up to four drying frames at once.

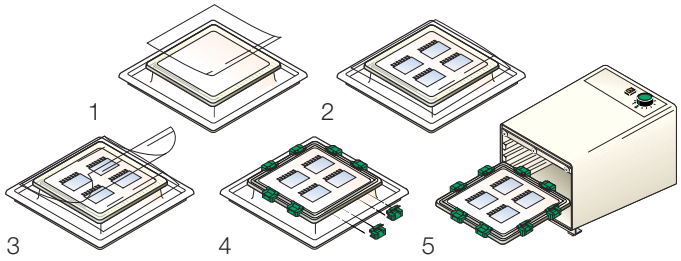

Fig. 12.4. Drying gels with the GelAir drying system. Step 1, place a frame on the assembly table and cover it with wet cellophane; 2, lay a gel on top of the cellophane; 3, place another sheet of wet cellophane over the gel; 4, clamp the drying frames together; 5, slide the drying frame into the dryer.

Gels can also be dried with a vacuum gel dryer. Bio-Rad's Model 583 gel dryer can dry multiple gels on its 35 x 45 cm surface. The system includes a heated lid and a gasket to ensure uniform application of vacuum. Gels are dried on a sheet of filter paper or a cellophane membrane support. The quiet water-cooled HydroTech™ pump provides a self-contained, constant vacuum.

Storage of Gels in Plastic Bags

As an alternative to drying gels, they can also be sealed in zip-top plastic bags. Gels are usually sealed in either water or, for long-term storage, water with 0.005% sodium azide. Fill the bag with water, then insert the gel, expel the water, and seal the bag.

For ordering information related to this section see page 49

Part III

Troubleshooting Guide

Troubleshooting Guide

Problem: No spots or fewer than expected spots detected on gel

1. **Not enough sample loaded.** Check the concentration of starting material by protein assay procedures (see page 30). Check that your protein assay is functioning properly and not responding to interfering substances in your sample. Consider what percentage of the sample loaded might be represented in each spot. For example, if you want to detect 1,000 spots, and your detection method can detect 10 ng per spot as a lower limit (SYPRO Ruby, Bio-Safe™ Coomassie, or Silver Stain Plus™ stains), you should load at least 100,000 ng (100 µg) of protein per gel if you expect most proteins to be 10x the abundance of your lower limit. If your detection method is less sensitive, or your protein of interest is of lower abundance, you must load more sample. See pages 7 and 32 for further information on protein load. A guideline for new samples is to adjust the total protein concentration in the rehydration solution to 1 mg/ml.
2. **Insufficient sample entered the IPG strip.** Insoluble proteins will not enter the IPG strip pores. Proteins can be solubilized by adjusting various components in the sample extraction solution, for example, detergent, reducing agent, and ampholyte concentration, as well as pH and ionic strength. See the sample preparation discussion (Chapter 2) for more thorough discussion of factors affecting solubility of proteins applied to IPG strips. Sample application methodology can also affect solubilization; a discussion on sample application is found on pages 10–11. See page 28 for enhanced solubilization solutions.
3. **pH gradient oriented incorrectly during focusing.** Check to make sure that the end of the IPG strip marked with a "+" is oriented toward the positive electrode.
4. **Detection method not sensitive enough.** Make sure that the detection method is sensitive enough to detect the amount of protein loaded. A titration may be helpful: Load strips with increasing concentrations of sample, focus, and stain the strips with Bio-Safe Coomassie Blue or IEF gel staining solutions. Select the concentration of subsequent loads from the load that has the most detectable proteins without distortion or severe overloading.
5. **Failure of detection reagents.** Loading a lane of unstained protein standards as described on page 37 can help to diagnose this problem. If the standards are not detected, you should check the expiration dates and the formulations of all detection reagents.

Problem: Streaking and smearing on 2-D gel

Horizontal streaks

Horizontal streaks indicate a problem with the IEF run. They can be caused by a number of factors, including liquid in excess of the amount absorbed by the strip, high viscosity, overloading, sample solubility problems, incomplete focusing, or nonproteinaceous material (especially nucleic acids) adhering to proteins.

1. **Sample preparation problems.** This is the most common cause of horizontal streaking. In this case, significant amounts of protein may also remain at the origin of the second-dimension gel. A new sample preparation method must be worked out for each type of sample. The concentations of urea, detergents, ampholytes, and reducing agents may be critical; see the section on solubilization on page 3. A literature search on preparation of proteins from the system of interest is a good place to start. If the sample is a membrane protein sample or a cell lysate, then high-speed centrifugation may help. The sample should be centrifuged at 100,000 x g after addition of IEF sample buffer (ampholytes, urea, detergent, reducing agent) even if it appears clear.

   An excellent reference is Molloy (2000).
2. **Too much protein loaded.** Try using 1/10 the amount of sample, or dilute the sample by at least one half. If your protein is not abundant, you may want to prefractionate your sample using the ReadyPrep™ sequential extraction kit, Rotofor® system, chromatography, or other procedure to enrich the protein of interest and lower the amounts of abundant proteins. See pages 7 and 32 for information on the amount of protein to load.
3. **Nucleic acids bound to protein.** Treatment with an endonuclease as discussed on pages 7 and 29 can reduce viscosity and improve protein absorption into the IPG strip. Make sure that the nuclease is active and that digestion is adequate. A very viscous sample implies that nuclease treatment has failed.
4. **Focusing time not optimized.** If focusing is not complete, proteins will not focus as tight spots. See pages 11 and 34.

Problem: Streaking and smearing on 2-D gel

Vertical streaks

Vertical streaks are related to second-dimension electrophoresis and are caused by various factors, including loss of solubility of a protein at its pI, dust contaminants, improper reducing agent, or incorrect placement of the IPG strips. These streaks can be analyzed like any other SDS-PAGE problem. For example, if SDS-PAGE standards have been applied next to the IEF strip on the second-dimension gel, and the standards do not have streaks, then the problem lies with the sample, rather than with the electrophoresis run.

1. **Pinpoint vertical streaks in the background.** These are most likely due to dust or particles in the water. The water purifier and storage containers should be checked for contamination. All gel solutions should be filtered through 0.45 µm nitrocellulose into a dust-free container.
2. **Vertical streaks, usually broad, connected to a spot.** Some streaks are above the spots, others are below the spots. If spots are streaky, some protein aggregation is indicated. Isoelectric point precipitation is difficult to predict and control. Some streaking is caused by incomplete reduction/alkylation. In such cases, an increased amount of reducing agent or changing the reducing agent to tributylphosphine (TBP) will help. In a small number of cases, increasing the SDS concentration during equilibration or increasing the time of equilibration will decrease streaking. Protein overloading can also cause vertical streaking.
3. **Twin vertical spots or vertical doublets throughout the gel.** This effect is sometimes seen when thiourea is included in the sample solution. Its cause is unclear.

Problem: Blank stripes in the vertical dimension

1. An air bubble may have been trapped in the agarose that joins the strip to the top of the second-dimension gel.
2. A region of the IPG strip may not have been sufficiently rehydrated or may have torn during handling, resulting in the absence of focused protein in that region.
3. Blank stripes near pH 7 are often caused by excessive DTT (>50 mM) in the IPG sample buffer.
4. Blank stripes at the electrodes, especially at the cathode, can be caused by a buildup of salt.

Problem: Known proteins showing up as multiple spots or at the wrong position

1. Proteins may be carbamylated if prepared too far ahead of time in urea, if exposed to high pH while in urea, or if allowed to exceed 30°C while in urea solutions.
2. Proteins may have been oxidized if the concentration of DTT is not sufficient. Many researchers prefer TBP for this reason; see page 5 for further discussion.
3. Proteolysis can be a problem if cells are harvested slowly or under warm conditions, especially in physiological buffers (lacking chaotropic agents). Use protease inhibitors, perform manipulations as quickly as possible, and keep solutions as cold as possible.

Problem: Spots absent on one side of the second-dimension gel

1. The IPG gradient range could be incorrect for your sample. For example, if all proteins in your sample have basic pI, and you are using an acidic gradient strip, then most of the proteins will focus to the basic side of the gel.
2. If you apply the sample after rehydration of the strip, then the movement of the proteins can be restricted if the gel is unevenly rehydrated.
3. Basic IPG strips (pH 7–10 and higher) generally give better results when cup loading is used.
4. Reverse endosmosis limits focusing to pH 11 in high-pH IPG strips.

Problem: No spots or fewer than expected spots in the high molecular weight regions

1. Sample may have been subject to proteolysis prior to focusing. Include appropriate protease inhibitors and keep the sample on ice or in a coldroom.
2. Equilibration steps between the first and second dimensions were not long enough. Make sure to incubate strips in sufficient volumes of each equilibration buffer for at least 10 min with mild agitation.
3. Poor entry of high molecular weight proteins during rehydration. The pore size of the acrylamide in the IPG strip is very small during the early stages of rehydration. Active sample loading in the focusing tray may help the entry of large proteins. See pages 10–11 for an in-depth discussion of sample application.

Problem: Problems during runs

1. **Focusing time too long or final voltage not reached.** One of the most important factors in IEF is to control the total ionic strength of the sample so that the current is kept low. With IPG strips, a low ionic strength sample is especially important. If the ionic strength is too high, the final voltage will not be reached. The optimum salt concentration is about 10 mM, although 40 mM can be tolerated. High conductivity can result from buffer salts in the sample, or from an inherently high conductivity of the sample (e.g., serum). We suggest you either desalt or dilute the sample so that the total conductivity is as low as possible. Ampholytes can often substitute for salts in the sample solution. We recommend a final concentration of 0.2% (w/v) ampholytes. Note that other commercially available IPG buffers may contribute conductivity to the sample.

   Many cell lysates must be desalted before they can be run on ReadyStrip™ IPG strips. A total ionic strength greater than 10 mM salt in the sample will increase your focusing time and could prevent the voltage from reaching the maximum setting. Salt will eventually electrophorese out of the strips. At 50 µA, it takes 4 hr for 40 mM salt to clear from a 17 cm strip. Use of wicks helps. It is also acceptable to increase the run time, giving as much time as necessary for the run to reach high voltage.

   If the sample has a high ionic strength, we recommend you remove the ions with Micro Bio-Spin™ 6 or Micro Bio-Spin 30 spin columns. These columns contain 10 mM Tris and they will remove all small molecules and ions from a protein sample with excellent protein recovery and no dilution of the sample.

   The simplest approach to focusing highly conductive samples is to be patient. Use electrode wicks and give the system sufficient time to reach high voltage.

2. **Arcing or burned IPG strips during focusing run.** Wicks should be used to absorb ionic contaminants that migrate to the ends of IPG strips during the run. Otherwise, the ions will collect at the electrodes and create regions of low resistance. Regions of low resistance create high current and high heat, leading to water evaporation, which may result in arcing or burning of the strip. The variable resistance error message will appear if you are using the PROTEAN® IEF cell.

   Whenever possible, we recommend no more than 10 mM total ionic strength in the sample. We suggest desalting with a column such as the Econo-Pac® 10DG desalting column or the Micro Bio-Spin column. The 10DG column is useful for samples between 1 and 3 ml. The Micro Bio-Spin column is useful for samples of ≤75 µl.

3. **Mineral oil migrating between wells in the PROTEAN® IEF focusing trays.** The sample does not migrate along with the oil. It is an aqueous solution and is not miscible with the oil. The oil will not migrate between wells unless the wells are overfilled or dirty. Note that proper cleaning of the trays eliminates the wicking problem (mineral oil migrating to next lane). We recommend cleaning in hot water with detergent and thorough rinsing (pay special attention to the corners). Note that any residual detergent left in the tray can also lead to wicking.

   The use of mineral oil is needed during IEF for two reasons:

- To prevent the gel from dehydrating during a long focusing run, which would cause urea to precipitate and possibly cause the strip to burn.
- To prevent CO_2 in the air from entering the gel and disrupting the pH gradient.

   However, oil does not necessarily need to be used in the rehydration trays. When oil is omitted, use the tray lid and seal the tray with plastic wrap or Parafilm.

4. **Interruption due to power failure.** If your laboratory is susceptible to power failures, we suggest an uninterruptible power supply for the PROTEAN IEF cell and other electronic instruments.

5. **Second-dimension run too long.** Long run times usually indicate a gel buffer that is too concentrated (conductivity is too high). The distribution of the applied voltage between the leading ions in the gel buffer and the trailing ions from the electrode buffer can slow protein migration and distort the gel pattern compared to gels run in properly made gel buffers.

6. **Second-dimension run too fast.** Short run times indicate that the gel buffer is too dilute (conductivity is too low). In this case, too, run time and gel pattern are a function of the distribution of the applied voltage between the gel and electrode buffers.

Part IV

Ordering Information

Ordering Information

Chapters 2 and 9 — Sample Preparation and Protein Assay

Catalog #	Description	Quantity	Page
163-2105	ReadyPrep™ 2-D Starter Kit, includes protein sample and reagents sufficient to rehydrate, focus, and transfer to the second-dimension gel six 17 cm, ten 11 cm, or sixteen 7 cm ReadyStrip™ IPG strips	1 kit	32
163-2103	ReadyPrep Sequential Extraction Kit Reagent 2, reconstitutes to 10 ml	1 vial	28–29
163-2106	ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer, reconstitutes to 10 ml	1 vial	27–28
163-2110	*E. coli* Protein Sample, lyophilized	2.7 mg	27
161-0730	Urea	250 g	3, 27–28
161-0731	Urea	1 kg	3, 27–28
161-0611	Dithiothreitol (DTT)	5 g	3, 27
163-2101	Tributylphosphine (TBP), 200 mM	0.6 ml	3, 27–29
161-0460	CHAPS	1 g	3, 27–29
161-0407	Triton X-100	500 ml	3
163-1112	Bio-Lyte® 3/10 Ampholyte, 40% (w/v)	10 ml	3, 27–29
163-1132	Bio-Lyte 3/5 Ampholyte, 20% (w/v)	10 ml	3, 27–29
163-1142	Bio-Lyte 4/6 Ampholyte, 40% (w/v)	10 ml	3, 27–29
163-1152	Bio-Lyte 5/7 Ampholyte, 40% (w/v)	10 ml	3, 27–29
163-1172	Bio-Lyte 7/9 Ampholyte, 40% (w/v)	10 ml	3, 27–29
163-1182	Bio-Lyte 8/10 Ampholyte, 20% (w/v)	10 ml	3, 27–29
163-1192	Bio-Lyte 5/8 Ampholyte, 40% (w/v)	10 ml	3, 27–29
163-2093	100x ReadyStrip 7–10 Buffer	1 ml	3, 27–29
163-2094	100x Bio-Lyte 3/10 Ampholyte	1 ml	3, 27–29
163-2095	100x ReadyStrip 6.3–8.3 Buffer	1 ml	3, 27–29
163-2096	100x ReadyStrip 5.5–6.7 Buffer	1 ml	3, 27–29
163-2097	100x ReadyStrip 4.7–5.9 Buffer	1 ml	3, 27–29
163-2098	100x ReadyStrip 3.9–5.1 Buffer	1 ml	3, 27–29
161-0404	Bromophenol Blue	10 g	27–28
161-0716	Tris	500 g	3, 27–29
161-0719	Tris	1 kg	27–29
142-6425	AG 501-X8 (D) Mixed Bed Resin	500 g	27
163-2104	ReadyPrep Sequential Extraction Kit Reagent 3, reconstitutes to 10 ml	1 vial	28
163-2100	ReadyPrep Sequential Extraction Kit, includes 1 vial of reagent 1 (to make 50 ml), 3 vials of reagent 2 (to make 10 ml each), 2 vials of reagent 3 (to make 10 ml each), 1 vial containing 0.6 ml of 200 mM TBP	1 kit	28
732-6701	Aurum™ Serum Protein Mini Kit, 10 preps, includes 10 serum protein columns, 10 clear 12 x 75 mm polystyrene tubes, 30 sample collection tubes, 10 column tips, 50 ml binding buffer, protocol overview, instructions	1 kit	5
500-0001	Bio-Rad Protein Assay Kit I, contains 450 ml dye reagent concentrate and a bovine γ-globulin standard, based on method of Bradford	1 kit	30
500-0121	*RC DC*™ Protein Assay Kit I, includes *RC* reagents package, *DC*™ protein assay reagents package, bovine γ-globulin standard, 500 standard assays	1 kit	31
170-2501	SmartSpec™ 3000 Spectrophotometer, UV/visible scanning spectrophotometer, 200–800 nm		30
Sigma E8263	Endonuclease, recombinant, from *Serratia marcescens*		7, 29
Sigma T8656	Thiourea (ACS Reagent Grade)		3, 28–29

Chapters 3 and 10 — Isoelectric Focusing

Catalog #	Description	Quantity	Page
	PROTEAN® IEF System		
165-4000	PROTEAN IEF System, complete, includes basic unit, 17, 11, and 7 cm focusing trays with lid, 1 pack each of 17, 11, and 7 cm rehydration/equilibration trays with lid, 2 pairs of forceps, pack of electrode wicks, mineral oil, cleaning brush	1 system	32–34
165-4001	PROTEAN IEF Cell, 90–240 VAC, basic unit, includes cell, instructions	1 unit	33–34
165-4050	Cup Loading Tray, includes 1 pair moveable electrodes, 1 pack each of large and small replacement cups	1 unit	10–11
165-4051	Large Replacement Cups	1 pack	10–11
165-4052	Small Replacement Cups	1 pack	10–11
165-4071	Electrode Wicks, precut	500	33
165-4035	Disposable Rehydration/Equilibration Tray With Lid, 7 cm	25 pack	32
165-4025	Disposable Rehydration/Equilibration Tray With Lid, 11 cm	25 pack	32
165-4015	Disposable Rehydration/Equilibration Tray With Lid, 17 cm	25 pack	32
165-4041	Disposable Rehydration/Equilibration Tray With Lid, 18 cm	25 pack	32
165-4043	Disposable Rehydration/Equilibration Tray With Lid, 24 cm	25 pack	32
165-4030	7 cm Focusing Tray With Lid	1	33
165-4020	11 cm Focusing Tray With Lid	1	33
165-4010	17 cm Focusing Tray With Lid	1	33
165-4040	18 cm Focusing Tray With Lid	1	33
165-4042	24 cm Focusing Tray With Lid	1	33

Chapters 3 and 10 — Isoelectric Focusing (cont.)

Catalog #	Description	Quantity	Page
165-4070	Forceps	1 pair	32–33
163-2129	Mineral Oil	500 ml	32–33
165-4080	Thermal Printer, 100 V, includes cable and power adaptor		34
165-4082	Thermal Printer, 120 V		34
165-4085	Thermal Printer, 220 V		34
	ReadyStrip™ IPG Strips		
163-2000	ReadyStrip IPG Strip, 7 cm, pH 3–10	12	32–33
163-2002	ReadyStrip IPG Strip, 7 cm, pH 3–10 (NL)	12	32–33
163-2001	ReadyStrip IPG Strip, 7 cm, pH 4–7	12	32–33
163-2003	ReadyStrip IPG Strip, 7 cm, pH 3–6	12	32–33
163-2004	ReadyStrip IPG Strip, 7 cm, pH 5–8	12	32–33
163-2005	ReadyStrip IPG Strip, 7 cm, pH 7–10	12	32–33
163-2028	ReadyStrip IPG Strip, 7 cm, pH 3.9–5.1	12	32–33
163-2029	ReadyStrip IPG Strip, 7 cm, pH 4.7–5.9	12	32–33
163-2030	ReadyStrip IPG Strip, 7 cm, pH 5.5–6.7	12	32–33
163-2031	ReadyStrip IPG Strip, 7 cm, pH 6.3–8.3	12	32–33
163-2014	ReadyStrip IPG Strip, 11 cm, pH 3–10	12	32–33
163-2016	ReadyStrip IPG Strip, 11 cm, pH 3–10 (NL)	12	32–33
163-2015	ReadyStrip IPG Strip, 11 cm, pH 4–7	12	32–33
163-2017	ReadyStrip IPG Strip, 11 cm, pH 3–6	12	32–33
163-2018	ReadyStrip IPG Strip, 11 cm, pH 5–8	12	32–33
163-2019	ReadyStrip IPG Strip, 11 cm, pH 7–10	12	32–33
163-2024	ReadyStrip IPG Strip, 11 cm, pH 3.9–5.1	12	32–33
163-2025	ReadyStrip IPG Strip, 11 cm, pH 4.7–5.9	12	32–33
163-2026	ReadyStrip IPG Strip, 11 cm, pH 5.5–6.7	12	32–33
163-2027	ReadyStrip IPG Strip, 11 cm, pH 6.3–8.3	12	32–33
163-2007	ReadyStrip IPG Strip, 17 cm, pH 3–10	12	32–33
163-2009	ReadyStrip IPG Strip, 17 cm, pH 3–10 (NL)	12	32–33
163-2008	ReadyStrip IPG Strip, 17 cm, pH 4–7	12	32–33
163-2010	ReadyStrip IPG Strip, 17 cm, pH 3–6	12	32–33
163-2011	ReadyStrip IPG Strip, 17 cm, pH 5–8	12	32–33
163-2012	ReadyStrip IPG Strip, 17 cm, pH 7–10	12	32–33
163-2020	ReadyStrip IPG Strip, 17 cm, pH 3.9–5.1	12	32–33
163-2021	ReadyStrip IPG Strip, 17 cm, pH 4.7–5.9	12	32–33
163-2022	ReadyStrip IPG Strip, 17 cm, pH 5.5–6.7	12	32–33
163-2023	ReadyStrip IPG Strip, 17 cm, pH 6.3–8.3	12	32–33

Chapters 4 and 11 — Second Dimension — Precast Gels

Catalog #	Description	Quantity	Page
	PROTEAN II Ready Gel® Precast Gels, IPG Well, for Use with 17 cm ReadyStrip IPG Strips		
161-1450	Ready Gel Tris-HCl Gel for PROTEAN II Cell, 10%, IPG well, 1.0 mm thick, 18.3 x 19.3 cm	each	14, 35
161-1451	Ready Gel Tris-HCl Gel for PROTEAN II Cell, 12%, IPG well, 1.0 mm thick, 18.3 x 19.3 cm	each	14, 35
161-1452	Ready Gel Tris-HCl Gel for PROTEAN II Cell, 10–20%, IPG well, 1.0 mm thick, 18.3 x 19.3 cm	each	14, 35
161-1453	Ready Gel Tris-HCl Gel for PROTEAN II Cell, 8–16%, IPG well, 1.0 mm thick, 18.3 x 19.3 cm	each	14, 35
	Criterion™ Precast Gels, IPG Well, for Use with 11 cm ReadyStrip IPG Strips		
345-0013	Criterion Tris-HCl Gel, 10% resolving, 4% stacking gel	each	14, 35
345-0018	Criterion Tris-HCl Gel, 12.5% resolving, 4% stacking gel	each	14, 35
345-0031	Criterion Tris-HCl Gel, 4–15%	each	14, 35
345-0036	Criterion Tris-HCl Gel, 4–20%	each	14, 35
345-0041	Criterion Tris-HCl Gel, 8–16% resolving, 4% stacking gel	each	14, 35
345-0046	Criterion Tris-HCl Gel, 10–20% resolving, 4% stacking gel	each	14, 35
	Ready Gel Precast Gels, IPG Well, for Use with 7 cm ReadyStrip IPG Strips		
161-1390	Ready Gel Tris-HCl Gel, 10% resolving, 4% stacking gel	each	14, 35
161-1391	Ready Gel Tris-HCl Gel, 12% resolving, 4% stacking gel	each	14, 35
161-1392	Ready Gel Tris-HCl Gel, 4–15%	each	14, 35
161-1393	Ready Gel Tris-HCl Gel, 4–20%	each	14, 35
161-1394	Ready Gel Tris-HCl Gel, 8–16% resolving, 4% stacking gel	each	14, 35
161-1395	Ready Gel Tris-HCl Gel, 10–20% resolving, 4% stacking gel	each	14, 35

Chapters 4 and 11 — Casting Multiple Gels — Equipment

Catalog #	Description	Quantity	Page
	Casting Multiple PROTEAN Plus™ Gels		
165-4160	PROTEAN Plus Multi-Casting Chamber, includes casting chamber, sealing plate, silicone gasket, tapered luer connector, leveling bubble, acrylic blocks, separation sheets, instructions (PROTEAN Plus combs and spacer plates must be ordered separately)	1	35
165-4121	Model 495 Gradient Former, includes body with valve stem and tubing connection kit	1	35
165-4170	PROTEAN Plus Hinged Spacer Plate, 20 x 20.5 (W x L) cm, 1.0 mm	1	35
165-4171	PROTEAN Plus Hinged Spacer Plate, 20 x 20.5 (W x L) cm, 1.5 mm	1	35
165-4172	PROTEAN Plus Hinged Spacer Plate, 20 x 20.5 (W x L) cm, 2.0 mm	1	35
165-4173	PROTEAN Plus Hinged Spacer Plate, 25 x 20.5 (W x L) cm, 1.0 mm	1	35
165-4174	PROTEAN Plus Hinged Spacer Plate, 25 x 20.5 (W x L) cm, 1.5 mm	1	35
165-4175	PROTEAN Plus Hinged Spacer Plate, 25 x 20.5 (W x L) cm, 2.0 mm	1	35
165-4176	PROTEAN Plus Comb, 2-D (1 reference well), 20 cm, 1.0 mm	1	35
165-4177	PROTEAN Plus Comb, 2-D (1 reference well), 20 cm, 1.5 mm	1	35
165-4178	PROTEAN Plus Comb, 2-D (1 reference well), 20 cm, 2.0 mm	1	35
165-4179	PROTEAN Plus Comb, 2-D (1 reference well), 25 cm, 1.0 mm	1	35
165-4180	PROTEAN Plus Comb, 2-D (1 reference well), 25 cm, 1.5 mm	1	35
165-4181	PROTEAN Plus Comb, 2-D (1 reference well), 25 cm, 2.0 mm	1	35
	Casting Criterion™ Gels		
345-9905	Criterion Empty Cassette, 1.0 mm thick with IPG comb	10 sets	35
	Casting Multiple Mini-PROTEAN® 3 Gels		
165-4110	Mini-PROTEAN 3 Multi-Casting Chamber, includes 8 acrylic blocks, 15 separation sheets, tapered luer connector, stopcock valve	each	35
165-4120	Model 485 Gradient Former, includes body with valve stem and tubing connection kit	each	35
165-3308	Short Plates	5	35
165-3311	Spacer Plates With 1.0 mm Integrated Spacers	5	35
165-3312	Spacer Plates With 1.5 mm Integrated Spacers	5	35
165-3362	Mini-PROTEAN 3 Comb, IPG well, 1.0 mm	2	35
165-3368	Mini-PROTEAN 3 Comb, IPG well, 1.5 mm	2	35
	AnyGel™ Stands		
165-4131	AnyGel Stand, single-row, holds 1 PROTEAN® gel, 2 Criterion gels, or 3 mini gels; includes instructions	1	36
165-5131	AnyGel Stand, 6-row, holds 6 PROTEAN gels, 12 Criterion gels, or 18 mini gels; includes instructions	1	36
165-3221	Mini-PROTEAN 3 Cell and Single-Row AnyGel Stand, includes 165-3301 and 165-4131	1	36
165-6020	Criterion Cell and Single-Row AnyGel Stand, includes 165-6001 and 165-4131	1	36

Chapters 4 and 11 — Casting Multiple Gels — Reagents

Catalog #	Description	Quantity	Page
161-0722	Cleaning Concentrate, 50x	1 kg	7, 35, 44
161-0798	Resolving Gel Buffer, 1.5 M Tris-HCl, pH 8.8	1 L	35
161-0799	Stacking Gel Buffer, 0.5 M Tris-HCl, pH 6.8	1 L	35
161-0158	30% Acrylamide/Bis Solution, 37.5:1	500 ml	35
161-0148	40% Acrylamide/Bis Solution, 37.5:1	500 ml	35
161-0149	40% Acrylamide/Bis Solution, 37.5:1	2 x 500 ml	35
161-0122	Acrylamide/Bis Powder, 37.5:1	30 g	35
161-0125	Acrylamide/Bis Powder, 37.5:1	150 g	35
161-0141	40% Acrylamide Solution	2 x 500 ml	35
161-0142	2% Bis Solution	500 ml	35
161-0202	Piperazine Diacrylamide (PDA; alternative crosslinker to bis)	10 g	35
161-0800	TEMED	5 ml	35
161-0801	TEMED	50 ml	35
161-0700	Ammonium Persulfate (APS)	10 g	35

Chapters 4 and 11 — Equilibration of Focused Strips for the Second Dimension

Catalog #	Description	Quantity	Page
165-4035	Disposable Rehydration/Equilibration Tray With Lid, 7 cm	25 pack	36
165-4025	Disposable Rehydration/Equilibration Tray With Lid, 11 cm	25 pack	36
165-4015	Disposable Rehydration/Equilibration Tray With Lid, 17 cm	25 pack	36
163-2108	ReadyPrep 2-D Starter Kit Equilibration Buffer II, without DTT or iodoacetamide, reconstitutes to 20 ml	1 vial	36
163-2107	ReadyPrep 2-D Starter Kit Equilibration Buffer I, with DTT, reconstitutes to 20 ml	1 vial	36
161-0730	Urea	250 g	36
161-0731	Urea	1 kg	36
161-0300	SDS	25 g	13, 36
161-0301	SDS	100 g	13, 36
161-0302	SDS	1 kg	13, 36
161-0418	SDS Solution, 20%	1 L	13, 36
161-0798	Resolving Gel Buffer, 1.5 M Tris-HCl, pH 8.8	1 L	13, 36
161-0611	Dithiothreitol (DTT)	5 g	36
163-2109	Iodoacetamide	30 g	36
Sigma	Glycerol		36

Chapters 4 and 11 — Running the Second Dimension

Catalog #	Description	Quantity	Page
	Reagents and Premixed Solutions		
163-2111	ReadyPrep Overlay Agarose	50 ml	36
161-3111	Certified™ Low-Melt Agarose	25 g	36
161-0404	Bromophenol Blue	10 g	36
161-0732	10x Tris/Glycine/SDS	1 L	36–37
161-0772	10x Tris/Glycine/SDS	5 L	36–37
	Running up to 12 PROTEAN Plus Gels or PROTEAN II Ready Gel Precast Gels		
165-4150	PROTEAN Plus Dodeca™ Cell, 100/120V, includes tank and lid, buffer recirculation pump with tubing	1 cell	14, 15, 37
165-4151	PROTEAN Plus Dodeca Cell, 220/240V, includes tank and lid, buffer recirculation pump with tubing	1 cell	14, 15, 37
	Running Dual PROTEAN XL Gels or PROTEAN II Ready Gel Precast Gels		
165-3188	PROTEAN II XL Cell, wide format, 1.0 mm	1 system*	14–15, 36–37
165-3189	PROTEAN II XL Cell, wide format, 1.5 mm	1 system*	14–15, 36–37
165-3190	PROTEAN II XL Cell, wide format, 2.0 mm	1 system*	14–15, 36–37
	*Each system includes PROTEAN XL basic unit with casting stand, upper and lower buffer chambers, cooling core, lid with cables, plates, spacers		
	Running up to 12 Criterion Gels		
165-4130	Criterion Dodeca Cell, includes tank and lid	1 cell	14–15, 36–37
	Running Dual Criterion Gels		
165-6001	Criterion Cell, includes tank, lid with power cables	1 cell	14–15, 36–37
	Running up to 12 Mini-PROTEAN 3 Gels or Ready Gel Precast Gels		
165-4100	Mini-PROTEAN 3 Dodeca Cell, includes 6 clamp assemblies, 2 buffer dams, drain line, 2 gel releasers	1 cell	14–15, 36–37
165-4101	Mini-PROTEAN 3 Dodeca Cell, with multi-casting chamber	1 cell	14–15, 35–37
	Running Dual Mini-PROTEAN 3 Gels or Ready Gel Precast Gels		
165-3302	Mini-PROTEAN 3 Electrophoresis Module, for Ready Gel precast gel applications	1 module	36–37
165-3337	Mini-PROTEAN 3 Casting Module, 1.0 mm, IPG comb	1 module	36–37
165-3343	Mini-PROTEAN 3 Casting Module, 1.5 mm, IPG comb	1 module	36–37

Chapters 5 and 12 — Protein Detection Methods — Gels

Catalog #	Description	Quantity	Page
	Detection in Gels		
161-0436	Coomassie Brilliant Blue R-250 Staining Solution	1 L	16, 38
161-0437	Coomassie Brilliant Blue R-250 Staining Solution	4 x 1 L	16, 38
161-0439	Coomassie Brilliant Blue R-250 Destaining Solution	4 x 1 L	16, 38
161-0786	Bio-Safe™ Colloidal Coomassie Blue G-250 Stain	1 L	16, 38
161-0787	Bio-Safe Colloidal Coomassie Blue G-250 Stain	5 L	16, 38
170-3125	SYPRO Ruby Protein Gel Stain	1 L	16, 38
170-3138	SYPRO Ruby Protein Gel Stain	5 L	16, 38
161-0443	Bio-Rad Silver Stain Kit	1 kit	17, 39
161-0449	Silver Stain Plus™ Kit	1 kit	17, 39
	Methanol, reagent grade		38
	Acetic Acid, reagent grade		38
165-1771	GelAir™ Drying System, 115 V, 60 Hz, includes dryer, 2 drying frames, 16 clamps, assembly table, 50 precut sheets of cellophane support, gel drying solution	1 system	39
165-1772	GelAir Drying System, 230 V, 50 Hz	1 system	39
165-1775	GelAir Drying Frames, includes 16 clamps	2	39
165-1779	GelAir Cellophane Support, precut sheets	50 sheets	39
161-0752	Gel Drying Solution	1 L	39
165-1789	HydroTech™ Gel Drying System, 100/120 V, includes Model 583 gel dryer, HydroTech vacuum pump	1 system	39
165-1790	HydroTech Gel Drying System, 220/240 V	1 system	39
165-0962	Filter Paper Backing, 35 x 45 cm	25 sheets	39
165-0963	Cellophane Membrane Backing, 35 x 45 cm	50 sheets	39

Chapters 6 and 12 — Protein Detection Methods — Blots

Catalog #	Description	Quantity	Page
	Apparatus and Accessories		
170-3940	Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell	1 cell	18
170-3939	Trans-Blot Cell with Plate Electrodes and Super Cooling Coil, includes 2 gel holder cassettes, cell with lid and power cables, fiber pads, blot absorbent filter paper	1 cell	18
170-4070	Criterion Blotter with Plate Electrodes, includes cell assembled with plate electrodes, lid with cables, 2 Criterion gel holder cassettes, filter paper pack, fiber pad pack, gel blot assembly tray, roller, sealed ice cooling unit, instructions	1 cell	18
165-5052	PowerPac™ 200 Power Supply, 100/120 V	1	18
165-5053	PowerPac 200 Power Supply, 220/240 V	1	18
161-0734	10x Tris/Glycine, to make 25 mM Tris, 192 mM glycine, pH 8.3 (to make Towbin buffer, add methanol)	1 L	18
161-0771	10x Tris/Glycine, to make 25 mM Tris, 192 mM glycine, pH 8.3 (to make Towbin buffer, add methanol)	5 L	18
161-0778	10x Tris/CAPS Buffer	1 L	18
161-0418	SDS Solution, 20%	1 L	18
	Methanol, reagent grade		18
	Mini-Blot Membranes and Papers		
170-3966	Extra Thick Blot Absorbent Filter Paper, for semi-dry blotting of mini gels	60 sheets	18
162-0186	Sequi-Blot™ PVDF Membrane, 7 x 8.4 cm	10 sheets	18
162-0216	Sequi-Blot PVDF/Filter Paper Sandwich, 7 x 8.5 cm (tank blotting)	20 pack	18
162-0217	Sequi-Blot PVDF/Filter Paper Sandwich, 7 x 8.5 cm (tank blotting)	50 pack	18
	Criterion Blot Membranes and Papers		
170-3967	Extra Thick Blot Absorbent Filter Paper, for semi-dry blotting of Criterion gels	60 sheets	18
162-0180	Sequi-Blot PVDF Membrane, 10 x 15 cm	10 sheets	18
162-0236	Sequi-Blot PVDF/Filter Paper Sandwich, 8.5 x 13.5 cm (tank blotting)	20 pack	18
162-0237	Sequi-Blot PVDF/Filter Paper Sandwich, 8.5 x 13.5 cm (tank blotting)	50 pack	18
	Large Blot Membranes and Papers		
170-3969	Extra Thick Blot Absorbent Filter Paper, for semi-dry blotting of PROTEAN XL-size gels	30 sheets	18
162-0182	Sequi-Blot PVDF Membrane, 20 x 20 cm	10 sheets	18
162-0184	Sequi-Blot PVDF Membrane, 24 cm x 3.3 m	1 roll	18
	Blot Detection Reagents		
170-6527	Colloidal Gold Total Protein Stain	500 ml	20
161-0400	Coomassie Brilliant Blue R-250 Stain	10 g	20
161-0786	Bio-Safe Coomassie G-250 Stain	1 L	20
170-3127	SYPRO Ruby Protein Blot Stain	200 ml	20
170-6490	Immun-Blot® Kit for Glycoprotein Detection	1 kit	20

Chapters 6 and 12 — Protein Detection Methods — Blots (cont.)

Catalog #	Description	Quantity	Page
	General Immunodetection Color Detection Systems		
170-6460	Goat Anti-Rabbit IgG (H + L) Alkaline Phosphatase and BCIP/NBT	1 kit/200 assays	19
170-6461	Goat Anti-Mouse IgG (H + L) Alkaline Phosphatase and BCIP/NBT	1 kit/200 assays	19
170-6462	Goat Anti-Human IgG (H + L) Alkaline Phosphatase and BCIP/NBT	1 kit/200 assays	19
170-6463	Goat Anti-Rabbit IgG (H + L) HRP and 4CN	1 kit/200 assays	19
170-6464	Goat Anti-Mouse IgG (H + L) HRP and 4CN	1 kit/200 assays	19
170-6465	Goat Anti-Human IgG (H + L) HRP and 4CN	1 kit/200 assays	19
	Immun-Star™ Alkaline Phosphatase (AP) Chemiluminescent Detection		
170-5010	Immun-Star-AP Goat Anti-Mouse Detection Kit	50 miniblots	19
170-5011	Immun-Star-AP Goat Anti-Rabbit Detection Kit	50 miniblots	19
170-5012	Immun-Star-AP Substrate Pack	50 miniblots	19
170-5013	Immun-Star-AP Goat Anti-Mouse Intro Kit	8 miniblots	19
170-5014	Immun-Star-AP Goat Anti-Rabbit Intro Kit	8 miniblots	19
	Amplified Detection Systems		
170-6412	Amplified Alkaline Phosphatase Immun-Blot Assay Kit, goat anti-rabbit IgG (H + L)-biotin	50 miniblots	19
170-8235	Opti-4CN™ Substrate Kit	50 miniblots	19
170-8237	Opti-4CN Goat Anti-Mouse Detection Kit	50 miniblots	19
170-8236	Opti-4CN Goat Anti-Rabbit Detection Kit	50 miniblots	19
170-8238	Amplified Opti-4CN Substrate Kit	50 miniblots	19
170-8240	Amplified Opti-4CN Goat Anti-Mouse Detection Kit	50 miniblots	19
170-8239	Amplified Opti-4CN Goat Anti-Rabbit Detection Kit	50 miniblots	19

Chapter 7 — Image Acquisition and Analysis

Catalog #	Description	Quantity	Page
	Image Acquisition Systems		
170-7850	Molecular Imager FX Pro Plus™ MultiImager System, PC, 100–240 V	1	21
170-7851	Molecular Imager FX Pro Plus MultiImager System, Mac, 100–240 V	1	21
170-7856	Molecular Imager FX Pro™ Fluorescent Imaging System, PC, 100–240 V	1	21
170-7857	Molecular Imager FX Pro Fluorescent Imaging System, Mac, 100–240 V	1	21
170-8010	VersaDoc™ Model 1000 Imaging System, PC, 100/240 V	1	21
170-8011	VersaDoc Model 1000 Imaging System, Mac, 100/240 V	1	21
170-8030	VersaDoc Model 3000 Imaging System, PC, 100/240 V	1	21
170-8031	VersaDoc Model 3000 Imaging System, Mac, 100/240 V	1	21
170-8050	VersaDoc Model 5000 Imaging System, PC, 100/240 V	1	21
170-8051	VersaDoc Model 5000 Imaging System, Mac, 100/240 V	1	21
170-7980	GS-800™ Calibrated Imaging Densitometer, PC	1	21
170-7981	GS-800 Calibrated Imaging Densitometer, Mac	1	21
	Image Analysis Software		
170-8603	PDQuest™ 2-D Image Analysis Software, PC		22
170-8611	PDQuest 2-D Image Analysis Software, Mac		22
170-8637	PDQuest Extended License Contract, entitles licensed software user to request upgrade to update software released during one year period of the contract; network floating licenses also available		22

Chapter 8 — Protein Spot Excision

Catalog #	Description	Quantity	Page
165-7042	ProteomeWorks™ Spot Cutter with Fluorescent Enclosure, Windows, includes viewer mode PDQuest software for basic excision; not included, licensed version PDQuest 2-D software	1	23
165-7043	ProteomeWorks Spot Cutter with Fluorescent Enclosure, Mac, includes viewer mode PDQuest software for basic excision; not included, licensed version PDQuest 2-D software	1	23
165-7009	ProteomeWorks Spot Cutter, Windows, includes viewer mode PDQuest software for basic excision; not included, licensed version PDQuest 2-D software	1	23
165-7039	ProteomeWorks Spot Cutter, Mac, includes viewer mode PDQuest software for basic excision; not included, licensed version PDQuest 2-D software	1	23

Part V

References and Related Literature

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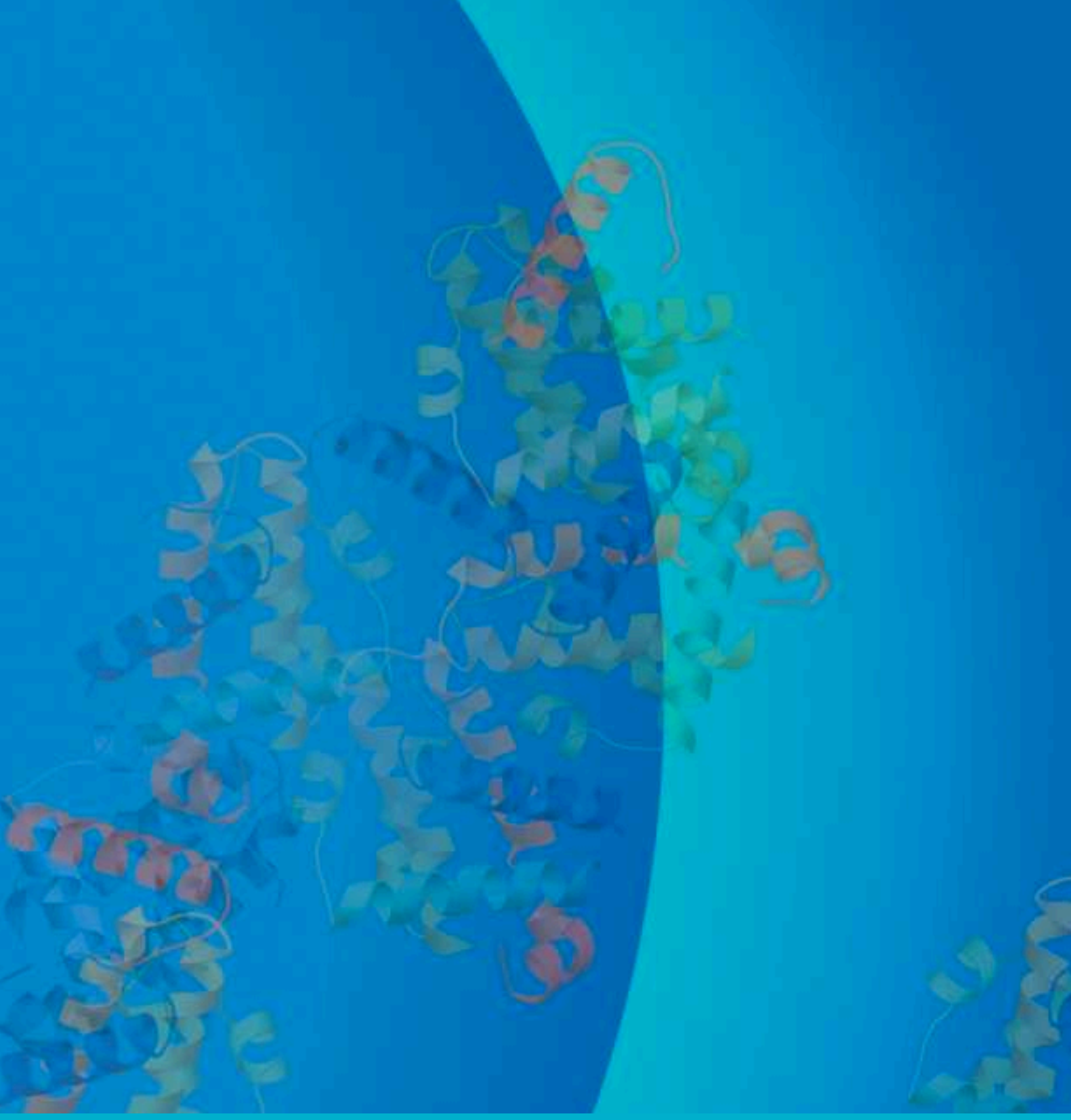

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