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Review

Staining methods in gel electrophoresis, including the use of multiple detection methods

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Abstract

Polyacrylamide gel electrophoresis is a reliable and widely used technique for the separation, identification and characterization of proteins and protein mixtures. With the introduction of high resolution two-dimensional polyacrylamide gel electrophoresis in 1975 upward to 2000 individual polypeptides spots are easily separated on a single electrophoretic gel thereby necessitating the availability of highly sensitive protein detection methods. Although a plethora of protein-staining and -visualization protocols have been described utilizing both radioactive and non-radioactive reagents, many times the use of mono-dimensional detection procedures is insufficient to address the experimental questions asked. The present review highlights the utilization of combined protein-labeling and -staining methodologies in gel electrophoresis including selected applications in polyacrylamide gels and solid membrane matrixes.

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1. Introduction

Polyacrylamide gel electrophoresis (PAGE) is a reliable and widely used technique for the separation, identification and characterization of proteins and protein mixtures. Since approximately 100 individual protein (polypeptide) bands and upward to 1000–2000 distinct polypeptide spots can easily be separated on standard one-dimensional (1D) PAGE and two-dimensional (2D) PAGE slab gels [1], respectively, the need for reliable and sensitive protein detection protocols is essential. Visualization of individual protein bands or spots is usually performed either in situ within the polyacrylamide gel matrix itself or following "Western" or electroblot transfer of separated proteins onto polymeric membrane support materials such as nitrocellulose, nylon or polyvinylidene difluoride membranes [2–5]. Post-electrophoretic, in situ, protein detection is by far the most frequently used method for both 1D- and 2D-PAGE applications although several procedures have been described for staining proteins prior to PAGE. Griffith's [6] method uses the reactive Remazol dyes for the staining of proteins upon denaturation before sodium dodecyl sulfate (SDS)-PAGE while Datyner and Finnimore [7] used a cationic dye of unspecified composition for the pre-staining of proteins prior to electrophoresis. While it is obvious that other reactive dyes could be used to stain proteins prior to electrophoresis, the post-electrophoretic staining of separated proteins is far and away the most preferred method and may involve one or more visualization procedures. These include organic [8] and inorganic metal salt based protocols [9–19], fluorescent group tagging [20], specific protein–ligand binding capacities [21], enzymic activity detection [22], as well as group-specific staining (e.g., glyco-, phospho-, lipoproteins, etc.) [23–27] and antibody immunostaining [20,28–40]. Alternatively, proteins which have been isotopically labeled with radioactive precursor molecules either prior to or post-electrophoretically can be visualized using autoradiographic and fluorographic detection on X-ray film [41–43]. The particular method of protein detection will, however, depend upon numerous factors including, most importantly, the experimental questions asked. Other considerations include the ease and reproducibility of the particular visualization procedure as well as the degree of sensitivity and accuracy of quantitation required. Although a myriad of protein-staining and -visualization protocols have been described (for reviews see [8,15,20,44–46] and references cited therein) none is totally ideal and often times the use of combined protein-staining and/or protein-labeling procedures is necessary.

2. In situ staining of proteins[1]

2.1. Choice of dyes

Proteins which are inherently colored such as hemoglobin and myoglobin are easily observed directly in polyacrylamide gels upon exposure to light in the visible spectrum. Unfortunately, this is not the case for the vast majority of proteins whose visualization requires the use of dyes or stains. Many of the organic dyes and stains that

[1] Any reference to a trademark or proprietary product does not constitute endorsement of that product by the United States Government and does not imply its approval to the exclusion of other products.

have been adapted for the detection of proteins in polyacrylamide gels have been derived from dyes originally utilized in the textile industry. Currently the most commonly used organic dyes include Amido Black 10B, Procion Blue RS, Ponceau S, Alcian Blue, Fast Green FCF, Coomassie Brilliant Blue R-250 (R = reddish hue) (CBB-R) and Xylene Cyanine Brilliant G (confusingly referred to as Coomassie Brilliant Blue G-250; G = greenish hue) (CBB-G). Recently, inorganic metallic salt-based staining or "shadowing" procedures have been developed that offer alternative methods of protein visualization. In particular, a plethora of silver-staining procedures have been developed that have been reported to be upward to a 100-fold more sensitive than the currently used CBB staining methods [9–19].

2.1.1. Organic dyes

2.1.1.1. Coomassie Brilliant Blue-based dyes

Of the commonly available organic dyes CBB-R and CBB-G are the most sensitive and convenient to use and as a result have become the most widely employed of the organic stains. CBB-R and CBB-G are chemically related aromatic compounds originally developed as acid wool dyes and named "Coomassie dyes" to commemorate the British occupation of the Ashanti capital, Kumasi or "Coomassie" now in Ghana [15]. Coomassie is a registered trademark held by the Imperial Chemical Industries (ICI) although ICI no longer produces CBB dyes. Numerous companies have obtained the rights to use the name "Coomassie" in addition to marketing equivalent CBB-based dyes under their own trademarks. These include Serva Blau (Blue) R or G, PAGE blue 83 (R) or 90 (G), Kenacid blue R, Supranocyanin 6B or G, Brilliant Blue R and Microme No. 1137 [8,46].

CBB-R is a non-polar, sulfated aromatic dye generally used in methanol–acetic acid solutions where excess dye is removed from the polyacrylamide gel matrix by destaining. CBB staining requires an acid environment to enhance ionic interactions between the dye and the basic amino acid moieties of the protein as well as to augment secondary dye–protein interactions due to hydrogen bonding, Van der Waals attraction, and hydrophobic bonding [47]. Hundreds of CBB staining recipes have been formulated containing dye, methanol, and acetic acid or trichloroacetic acid (TCA) and these have been reviewed by Righetti [48]. Neuhoff et al. [49] have comprehensively investigated over 600 variations in CBB staining/destaining procedures optimizing conditions with respect to staining sensitivity and linearity of staining response.

A typical CBB staining solution routinely used in our laboratory consists of 0.1–0.2% (w/v) CBB-R in 45% (v/v) methanol and 10% aqueous acetic acid which is filtered through a Whatman No. 1 filter paper immediately prior to use to remove any insoluble material. Duration of staining is dependent upon gel thickness and polyacrylamide composition, staining time increasing with gel thickness and polyacrylamide composition and decreasing with temperature. In practice, it is most convenient to stain gels overnight at room temperature with gentle shaking and destain the next day. Gels are destained using either passive diffusion or electrophoretic destaining. Passive diffusion destaining is the most commonly utilized method in which stained gels are placed in aqueous solutions of 30% (v/v) methanol and 10% acetic acid and gently agitated. Fresh destaining solution is replaced as the excess CBB leaches from the gels. After destaining, gels can be stored in 7.5% aqueous acetic acid in which the dye–protein complex is fixed and the color relatively stable. Because of the anionic nature of CBB, gels can also be destained electrophoretically using commercially available destaining apparatuses [50].

An alternative, more rapid, CBB-based staining procedure has been described using CBB-G, a dimethylated derivative of CBB-R. CBB-G is suspended in aqueous TCA in which it is relatively insoluble. Incubation of gels in the colloidal suspension of CBB-G results in the formation of dye–protein complexes, in which CBB-G does not penetrate the polyacrylamide gel matrix and interacts only to protein thus minimizing background staining. A typical procedure is to prepare a 0.05% solution of CBB-G in aqueous

methanol which is then diluted with an equal volume of 25% aqueous TCA. For optimal results fresh staining solution should be prepared each time. Major protein bands stain within 5–10 min. For complete staining of minor protein bands gels should be left in the staining solutions for several hours to overnight followed by destaining in 5% TCA.

Wilson [8] and Reisner et al. [51] have described modifications to this procedure in which a 0.04% (w/v) solution of CBB-G is prepared in 3.5% perchloric acid. Gels are immersed in sufficient staining solution such that the ratio of the volume of water in the gel to that of staining solution is 3:5. In 3.5% perchloric acid CBB-G is orange but upon binding to protein it changes to blue. Staining is very rapid and fairly abundant protein bands become visible within 10 s with most bands becoming visible within 10 min. Since only protein is stained background destaining is unnecessary. Sensitivity can be increased 3-fold by placing the gels in 5% aqueous acetic acid after staining at which time band color intensifies and the background becomes pale blue. For long term storage gels may be placed in a 3.5% perchloric acid solution containing 0.005% CBB-G. This method per se is not suitable for SDS-PAGE because of the binding of the SDS to proteins which markedly interferes with dye–protein interactions.

2.1.1.2. Miscellaneous organic dyes

In addition to the CBB-based staining techniques a large number of alternative protein-staining procedures have been described [20]. Many of these have been developed for specialized purposes and only a few are routinely used at present.

Historically, Amido Black 10B (Buffalo black NBR, naphthalene black 12B, aniline blue black, naphthol blue black, acid black 1, amido schwartz) was probably the first dye used to stain proteins in polyacrylamide gels; however, its use today is less frequent because of the availability of more sensitive CBB-based protocols. Nonetheless Amido Black still enjoys selected applications because of its rapid staining and destaining properties. Gels are gently shaken in 5–10 volumes of 7% (v/v) aqueous acetic acid containing 0.1% (w/v) Amido Black for a least 2 h to overnight. Gels are destained in 7% acetic acid with frequent changes. Earlier reports in the literature stating that Amido Black stains proteins metachromatically, frequently resulting in gels which contain bands of many shades of black, brown, red-brown and blue, were probably due to impurities in the dye [52].

Gorovsky et al. [53] have described a simple method for protein staining with Fast Green FCF (food green 3) which appears to be quite useful in quantitative densitometry. Fast Green FCF exhibits a greater linearity in staining intensity up to 150–200 μg/6 mm gel as compared to CBB-R. An additional property of Fast Green FCF is its capacity to form complexes with histones to give stable colors thereby making it a useful group-specific stain [28]. Fast Green FCF can also be used for staining of proteins in isoelectric focusing (IEF) gels. In contrast to CBB-R and CBB-G, Fast Green does not bind to ampholytes (Bio-Rad) thus staining only proteins [54]. Fig. 1 illustrates the relative staining sensitivities of CBB-R, CBB-G, Amido Black and Fast Green FCF for a group of standard protein preparations.

More recently, Hong et al. [55] have reported a protein-staining procedure in SDS-PAGE gels comparable in sensitivity to CBB staining utilizing 1-(2-hydroxy-4-sulfo-1-naphthylazo)-2-hydroxy-3-naphthoic acid (calconcarboxylic acid NN). The procedure can be performed using both simultaneous as well as post-electrophoretic staining techniques. Simultaneous staining using 0.01% NN in the upper reservoir buffer eliminates the post-staining step, and thus allows the detection of proteins more rapidly and simply. NN staining can detect as little as 10 ng of bovine serum albumin (BSA) by post-staining and 25 ng by simultaneous staining, compared to 50 ng detectable by CBB post-staining [55]. Of the dyes that display certain group-specific staining properties, "Stains-all", a cationic carbocyanine dye, has been shown to stain sialoglycoproteins and phosphoproteins blue and almost all other proteins red [26,27].

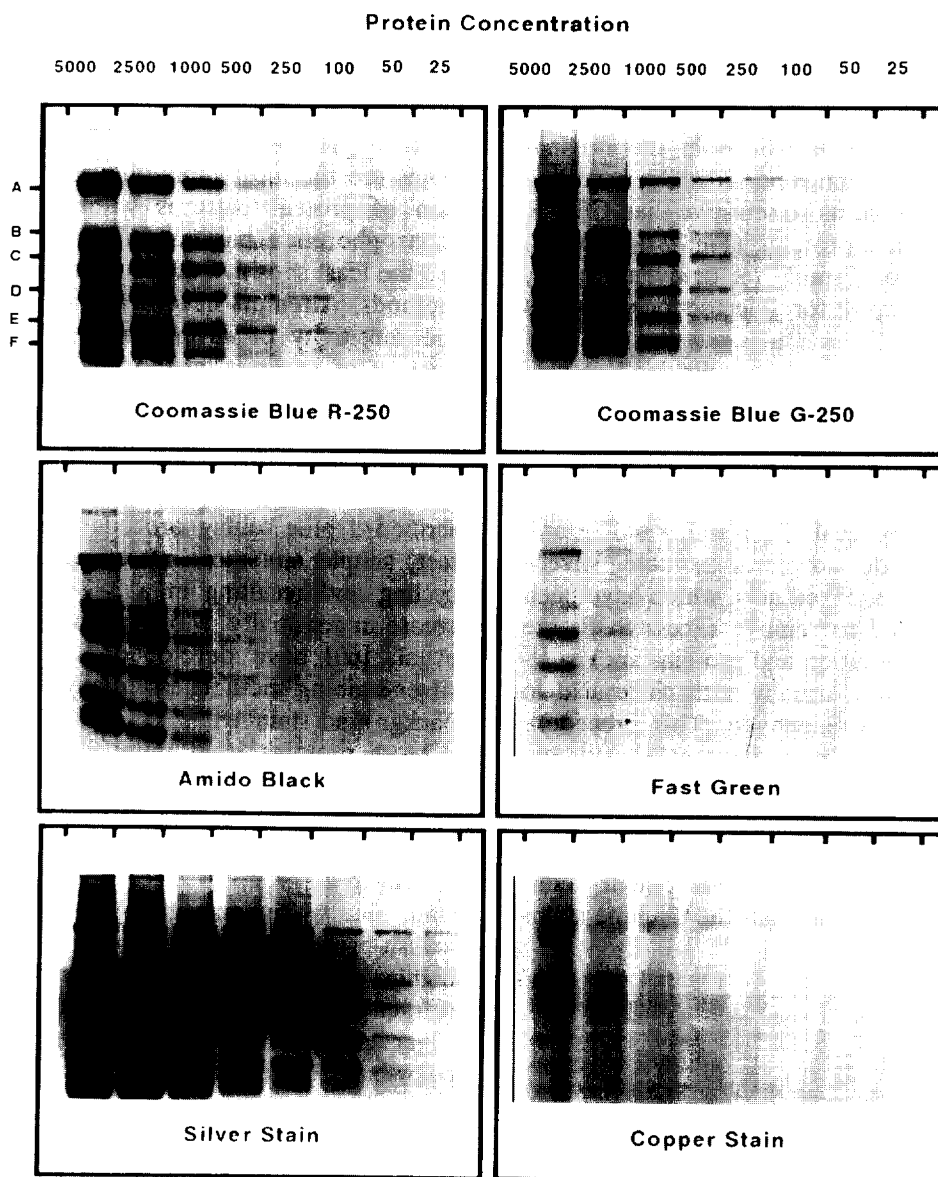

Fig. 1. Comparison of staining sensitivity of commonly used dyes. Bio-Rad protein standards: A = myosin (M_r 200 000); B = β-galactosidase (M_r 116 000); C = bovine serum albumin (M_r 66 000); D = ovalbumin (M_r 45 000); E = carbonic anhydrase (M_r 31 000); F = soybean trypsin inhibitor (M_r 21 500) were separated on 10% precast Tris-glycine Novex gels and stained as described in text. Protein concentrations ranged from 25 to 5000 ng/well.

2.1.2. Silver staining

For most applications, visualization of proteins with CBB is sufficiently sensitive. However, if one is interested in determining the absolute purity of a protein or wishes to determine trace amounts of proteins then more sensitive protein-visualization techniques must be utilized. With the possible exception of the autoradiographic and fluorographic detection of radiolabeled proteins, silver staining offers the greatest sensitivity in non-radioactive protein detection. Silver staining was first developed for histological purposes

[56] and later combined with photographic techniques for the detection of proteins and other biopolymers (e.g., DNA and RNA) in polyacrylamide matrixes in 1979 by Switzer et al. [9]. Since then over 100 variations in the original methodology have been reported. Silver staining is upward to 100-fold more sensitive than CBB-R staining for certain proteins (Fig. 1) [9–20,45] with sensitivity comparable to, or even greater, than autoradiography for selected polypeptides as shown in Fig. 2.

Silver-staining or silver-"shadowing" procedures can be divided into three basic categories: (a) diamine or ammoniacal silver stains, (b) chemically developed non-diamine type and (c) photoreduction silver stains [11,12,15]. The diamine or ammoniacal silver stains utilize ammonium hydroxide to form soluble silver–diamine complexes. Fixed gels, which have been extensively washed to remove excess SDS, are incubated in this solution and proteins are visualized by acidification, usually with citric acid in the presence of formaldehyde. This procedure originally developed by Switzer et al. [9] and modified by Oakley et al. [16] and Wray et al. [17] is particularly good for the staining of proteins separated on gels thicker than 1 mm. The non-diamine chemical development stains are generally more rapid than the diamine stains

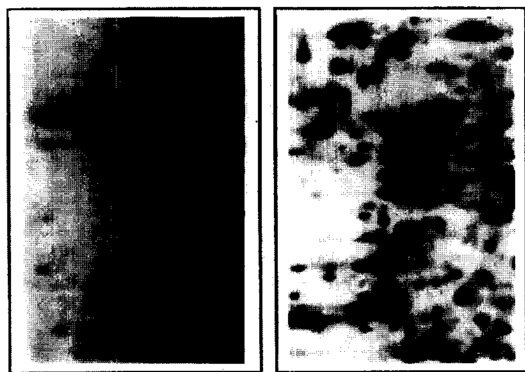

Fig. 2. Comparison of [^{35}S]methionine labeling (left) versus silver staining (right). Duplicate samples of [^{35}S]methionine-labeled RLE polypeptides (800 000 dpm, 100 μg protein) were separated using 2D-PAGE. One gel was silver stained while the other gel was dried and exposed to Kodak SB-5 film for 7 days at room temperature.

and are best suited for gels 1 mm or thinner. Image development of non-diamine stains occurs as a result of selective reduction of silver ions to elemental metallic silver by formaldehyde under alkaline pH [18,19]. The photoreduction silver stains are the most rapid allowing the visualization of protein patterns within 10 min after electrophoretic separation [57,58]; however, they are the least sensitive of the silver-based staining methods. Attempts have been made to increase the sensitivity of silver staining by the introduction of various "enhancing" or "recycling" steps. It is possible to recycle the stained gels through the procedure again from the silver nitrate treatment stage thus significantly increasing spot intensities [11]. Berson [59] has described a blue toning procedure claimed to be fast, simple and more effective than silver recycling and resulting in a 3–7-fold further increase in sensitivity. More recently Hochstrasser et al. [60] have developed a monochromatic silver-staining method essentially devoid of any background staining.

Most proteins stain monochromatically with silver yielding brown or black bands although certain silver stains can produce varying shades of black, blue, brown, red and yellow [19] which appear to be group specific. Lipoproteins tend to stain blue while some glycoproteins appear yellowish-brown or red [61]. Color formation is highly dependent upon silver-grain size, the refractive index of the gel and the distribution of silver grains in the gel although standardized color-based silver-staining kits are commercially available.

Although silver staining is the most sensitive of all the non-radioactive protein-visualization methods currently available it does have a number of drawbacks [46]. Probably one of the most serious problems, and one that deserves mention, is the fact that certain proteins stain either very poorly or not at all with silver, appearing as negatively stained spots against a darker background as illustrated in Fig. 3. This shortcoming is further emphasized by the lack of staining of certain calcium-binding proteins, such as calmodulin [62] and troponin C [63]. This lack of silver stainability was not characteristic of all

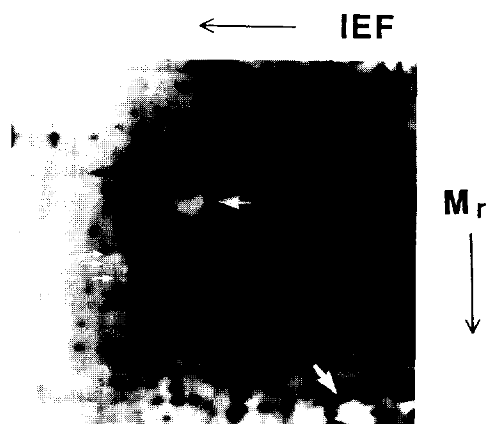

Fig. 3. Negative polypeptide staining using silver stain. Section of 2D-PAGE gel of RLE polypeptides stained with silver [60]. White arrows illustrate negatively stained polypeptides.

calcium binding proteins since parvalbumin stains strongly with silver [62].

2.1.3. Fluorescent protein labeling

Fluorescent methods for protein visualization following electrophoretic separation are extremely sensitive but are generally used less frequently than the CBB-based staining protocols due mainly to their relative complexity of use (e.g, requires ultraviolet illumination for protein visualization and sophisticated equipment for quantitation) and increased cost. Proteins are usually pre-labeled with a fluorescent sensitive dye prior to electrophoresis although proteins can be labeled post-electrophoretically as well. In most cases the introduction of these fluorescent tags into proteins involves a covalent interaction of the dye with terminal α-NH_2 and ϵ-NH_2 groups of lysines of the proteins thus modifying the net overall charge (p*I*) of the labeled molecules. In conventional SDS-PAGE this effect is insignificant since the mobility of a protein depends predominantly upon its molecular mass and the dye molecules are usually too small to produce an appreciable effect [64,65]. However, if one is performing 2D-PAGE analysis then pre-labeling with fluorescent tags could markedly alter protein migration in the first-dimension IEF mode thus precluding the use of labeled proteins as markers for the location of unlabeled molecules [1].

Fluorophores most commonly utilized to label proteins prior to electrophoresis include 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) chloride [66–68], 4-phenylspiro[furan-2(3H)-1′-phthalan]-3,3′-dione (fluorescamine) [69], 2-methoxy-2,4-diphenyl-3-(2H)-furanone (MDPF) [64], N-(7-dimethylamino-4-methylcoumarinyl)-maleimide (DACM) [70] and *o*-phthaldialdehye (OPA) [71]. Although dansyl chloride was the first fluorescent dye used for pre-staining proteins prior to electrophoresis subsequent studies have utilized fluorescamine since neither fluorescamine nor its hydrolysis products are fluorescent [20]. Fluorescamine has proved capable of detecting as little as 6 ng of myoglobin [72] with MDPF being 2.5 times more sensitive than fluorescamine with a linear response from 1 to 500 ng [64].

Proteins can also be detected post-electrophoretically with such reagents as 1-anilinonaphthalene-8-sulfonate (ANS) [73], Bis-ANS [74], fluorescamine [75], *p*-hydrazinoacridine [76] and OPA [44]. Since labeling with these reagents is usually performed under non-denaturing conditions they can be used quite advantageously for the rapid detection of proteins during preparative electrophoresis. Generalized procedures for both pre- and post-labeling with fluorescent dyes have appeared in reviews by Andrews [44], Hames and Rickwood [20] and Merril [15].

2.1.4. Reverse staining

In contrast to the "positive-staining" procedure described above, alternative less sensitive staining procedures based on the formation of insoluble metal (zinc, copper, potassium, etc.) salts have also been described. These methods, commonly referred to as "negative or reverse staining", are limited to SDS-containing gels and produce a semi-opaque background on the gel surface while the proteins are detected as transparent bands or spots when viewed against a black background or when properly backlighted [77–82]. Staining procedures are rapid (within 15 min), display intermediate sensitivity between

that of CBB staining and silver staining, do not require prior fixation of the proteins within the gel matrix, and since proteins remain unstained they are more readily recovered from gels (<90%) for subsequent biochemical characterization including Western immunoblotting, amino acid analysis and Edman N-terminal microsequencing. A representative copper-stained gel is illustrated in Fig. 1.

3. Labeling of proteins with radioactive isotopes

3.1. Radiolabeling methods

Labeling of proteins either prior to or post-electrophoretically using radioactive isotopes still remains the most sensitive method available for protein detection. The most commonly used isotopes include ^{14}C, ^{35}S, ^{32}P, ^{3}H and ^{125}I, although metal isotopes such as ^{59}Fe [83], ^{45}Ca [84], ^{63}Ni [85] and ^{75}Se have been used to identify iron, calcium and nickel binding proteins and Se–cysteine containing proteins, respectively. Whereas [^{32}P]orthophosphate has traditionally been the reagent of choice for the in vitro introduction of radioactive phosphate groups into proteins substitution of [^{33}P]- for [^{32}P]orthophosphate has gained increased popularity because of significantly increased polypeptide spot resolution and sharpness as well as increased safety factors afforded by use of the lower energy emitter ^{33}P as compared to ^{32}P [86]. Proteins can be radiolabeled any number of ways including both biosynthetic and chemical methods. A comprehensive summary of the procedures available is beyond the scope of this treatise and readers are referred to recent reviews by Dunbar [87], Andrews [44] and Parker [88].

3.1.1. Metabolic labeling of cells

In vitro metabolic labeling of cells or tissue sections in short-term culture by incorporation of isotopically labeled amino acid(s) precursor molecules during the cellular growth phase is routinely performed using either [^{3}H]leucine or [^{35}S]methionine. The use of [^{35}S]methionine is more favored because of its relatively higher energy β emitter potential (0.167 vs. 0.018 MeV), higher specific activity (>1000 vs. 50 Ci/mmol) and lower cost relative to [^{3}H]leucine. The extent of incorporation of [^{35}S]methionine is, however, dependent upon methionine content of the individual proteins and any proteins lacking methionine would be undetected using [^{35}S]methionine labeling. This problem has been circumvented by use of [^{14}C]amino acid-labeling mixtures [89,90]. In practice, however, this method is less favored due to the significantly lower specific activity of the [^{14}C]amino acid mixture (50 mCi/mmol) as well as the significantly higher cost of ^{14}C- versus ^{35}S-labeled amino acids. Proteins which are post-translationally modified via phosphorylation and glycosylation can be labeled with [^{32}P]/[^{33}P]orthophosphate and [^{3}H]/[^{14}C]galactose, mannose, N-acetylglucose- and galactoseamine (carbohydrates), respectively, while lipoproteins and certain membrane-associated polypeptides can be labeled with [^{3}H]/[^{14}C]palmitate and myristilate.

3.1.2. Post-synthetic radiochemical labeling

Alternatively, proteins can be labeled post-synthetically by any of a variety of chemical methods including oxidative iodination (tyrosine/histidine moieties and N-terminal or lysine residues), alkylation and acetylation (cysteine/serine/threonine residues) or reductive methylation (N-terminal amino or lysine residues) and these have been summarized by Andrews [44] and Rickwood [91]. Although both [^{125}I]- and [^{131}I]iodine have been used, ^{125}I is preferred for protein labeling because ^{125}I has a longer half-life) than ^{131}I (60 days versus 8 days for ^{131}I) and is safer to use than ^{131}I since the γ-rays of ^{125}I are much less penetrating than the β radiation of ^{131}I [88]. Alkylation and acetylation of hydroxyl and thiol group containing proteins is usually performed with ^{14}C-labeled α-haloacetic (iodo-, bromo-, chloro-) acid derivatives with α-iodoacetic acid or iodoacetamide being the most reactive. Reductive methylation of free amino groups with [^{14}C]formaldehyde and sodium cyanoboro[^{3}H]hydride is a frequently used and

relatively mild method with little or no deleterious effects on the labeled proteins [91].

3.1.3. Radioactive stains

The use of radioactive stains for the in situ detection of proteins has found limited applications because of the availability of relatively few radiolabeled reagents. In one application, however, [^{59}Fe]ferrous bathophenanthroline [92] has been used to radioactively label a series of protein markers in polyacrylamide gels post-electrophoretically using simple staining and destaining procedures [93].

3.2. Radioactivity detection methods

Individual radiolabeled protein bands or spots are usually detected one of three ways: liquid scintillation counting, autoradiography, and fluorography [41–44,94]. By far the most utilized method is that of autoradiography or variations developed to specifically increase the sensitivity of detection of proteins expressed at very low concentrations or for the detection of proteins labeled with low energy β-particle emitting radioisotopes, such as ^{3}H. These modifications include indirect autoradiography which utilizes intensifying screens for signal enhancement and fluorography [42,43,94]. Alternatively, following electrophoresis gels can be sectioned and radioactivity in individual gel pieces determined using liquid scintillation counting [44]. For practical purposes this method is not amendable for the analysis of 2D-PAGE gels and is limited to the analysis of proteins separated by 1D-slab or tube gel electrophoresis. The degree of resolution is dependent on band separation size which greatly limits its use. More recently, however, the technique of storage phosphor imaging has been introduced which has been reported to have a dynamic range of more than five orders of magnitude and a 10-fold greater sensitivity than autoradiography [95,96].

3.2.1. Autoradiography

Following electrophoresis radiolabeled proteins are most easily detected using autoradiography [41]. Dried polyacrylamide gels are placed in direct contact with appropriate X-ray film (e.g., Kodak X-Omat AR, Kodak SB-5, Kodak XAR, Kodak Direct exposure medical film, Fuji RX) where radioactive emissions (β-particles and/or γ-radiation) react with the silver halides in the film emulsion resulting in the formation of elemental silver atoms which are visualized following photographic development of the films. ^{14}C-, ^{35}S-, ^{32}P-, ^{125}I- and ^{131}I-labeled proteins are readily detected using direct autoradiography while ^{3}H-labeled proteins are very inefficiently detected due to severe quenching of their low energy β emissions by the polyacrylamide gel matrix.

3.2.2. Fluorography and indirect autoradiography

Because of the limitations in the detection of proteins labeled with low energy β-type emitters such as ^{3}H as well as the detection of proteins expressed in very minor amounts (e.g., transcription factors, single copy gene products, etc) fluorographic and indirect autoradiographic methodologies have been introduced [42,43,94]. Both procedures provide enhanced autoradiographic imaging of low to medium energy β-particle emitters (^{3}H, ^{14}C or ^{35}S) and involve the conversion of the emitted energy from the respective isotopes to photons of visible light, which become the predominant exposing radiation. This is accomplished either by the incorporation of an organic scintillator such as 2,5-diphenoxyoxazole (PPO) directly into the polyacrylamide gel matrix prior to fixation, drying and expose to film (fluorography) or by the use of calcium tungstate X-ray intensifying screens (indirect autoradiography). For optimal sensitivity film exposure utilizing X-ray intensifying screens such as Kodak X-OMATIC and DuPont Cronex Lightning Plus or Cronex Quanta II should be performed at very cold temperatures (-70°C) to stabilize latent image formation. This results in a 10–30-fold increase in the detection of ^{125}I and 8–10-fold increase in sensitivity to ^{32}P as compared to autoradiography at room temperature [97]. Additional rare earths (europium-activated barium fluorochloride or terbium-activated mixtures of lanthanium oxy-

sulfide and gadolinium oxysulfide) are available which appear to be more efficient that calcium tungstate for ^{32}P detection but result in higher background film darkening. The sensitivity of fluorography can be further increased by "pre-flashing" or hypersensitizing the film before main exposure [43]. This step has the added benefit of correcting the so-called "toeing" effect or non-linear relationship between radioactivity in sample to absorbance of the film image thus permitting quantitative measurements [43]. Table 1 summarizes the relative sensitivities of the various radioisotopic detection methods utilized for protein detection in polyacrylamide gels.

3.2.3. Storage phosphor imaging

Two of the most serious limitations to the use of X-ray film for the visualization of isotopically labeled proteins are (1) relative lack of sensitivity to β radiation and (2) a non-linear, limited dynamic range of film darkening to radiation exposure response [98]. An alternative methodology to the use of X-ray film for the detection and quantification of autoradiographic images has been introduced utilizing photostimulable storage phosphor imaging technology [96]. Storage phosphor imaging, which has found extensive use in clinical radiology, exhibits a dynamic exposure range of more than five orders of magnitude (100 000:1 versus 300:1 for X-ray film) and a 10–250-fold greater sensitivity than autoradiography to β-emissions [95,96]. Imaging screens are composed of a thin layer of $BaFBr:Eu^{2+}$ crystals in an organic binder [99]. Dried gels containing radiolabeled proteins are exposed to these plates in the same manner as X-ray film is exposed. Incident radiation (β-particles, γ-rays, X-rays, etc) emitted from labeled proteins induces excitation of the Eu^{2+} ions in the phosphor complex storing this excitation energy as a latent image. The latent images are scanned with a helium–neon laser which releases the store energy as blue photons and the intensity of luminescence is quantitated. ^{14}C, ^{35}S, ^{32}P, ^{33}P, ^{125}I, and ^{131}I are readily detected and quantitated. Fig. 4 illustrates a phosphoimage (A) and a corresponding autoradiogram (B) of a section of a Western immunoblotted 2D-PAGE

Table 1
Sensitivities of methods for radioisotopic detection in polyacrylamide gels

Isotope (MeV)[a]	Detection method	Sensitivity [dpm/cm^2 required for detectable image (e.g., A_{540} = 0.02) in 24 h]	Enhancement over direct autoradiography
^3H (0.018)[a]	Autoradiography	$>1700 \cdot 10^3$	–
^{14}C (0.156)	Autoradiography	6000	–
^{35}S (0.167)	Autoradiography	6000	–
^{125}I (0.035)	Indirect autoradiography (intensifying screens)	100	16
^{32}P (1.710)	Indirect autoradiography (intensifying screens)	50	10
^3H	Fluorography[b]	8000	>1000
^{14}C	Fluorography	400	15
^{35}S	Fluorography	400	15

Adapted from Laskey [94] and Hahn [41] The data are for exposure at −70°C using X-ray film pre-exposed to A_{540} = 0.15 above the background of unexposed film.
[a] Energy in MeV (^3H, ^{14}C, ^{35}S, ^{32}P β-emitters: ^{125}I γ-emitter) [41].
[b] PPO-impregnated gels.

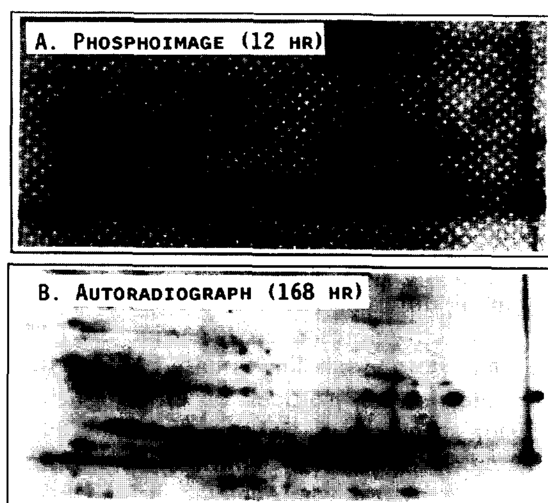

Fig. 4. Comparison of phosphoimage versus autoradiograph. Cellular lysates from epidermal growth factor treated National Institutes of Health (NIH) EGF-R cells were incubated with anti-phosphotyrosine antibodies and the immunoprecipitate separated using 2D-PAGE. The separated proteins were electroblotted to Immobilon PVDF membrane and incubated sequentially with anti-phosphotyrosine antibody and [^{125}I]protein A. Autoradiography was performed for 168 h at room temperature using Kodak XAR film while phosphoimage analysis was performed for 12 h using Kodak phosphor imaging plates and scanned on a Molecular Dynamics 400E PhosphorImager.

gel of immunoprecipitated phosphotyrosine polypeptides detected using antiphosphotyrosine antibodies and [^{125}I]Protein A. The phosphoimage is obtained after an overnight exposure while the autoradiogram was exposed for seven days at −70°C. The dynamic range of exposure was 0.11–10 000 counts for the phosphoimage (data not shown).

4. Multiple labeling strategies for protein characterization

SDS-PAGE and IEF are excellent methods for determining the purity as well as certain chemicophysical properties of specific proteins, such as molecular mass and isoelectric point (p*I*) but provide little or no information concerning either the biophysical or biological nature of the separated proteins. The use of ancillary analytical techniques subsequent to electrophoresis are therefore required for further biochemical characterization of the separated proteins. Functional group staining [23–27] as well as the staining for specific enzymatic activities directly on both 1D- and 2D-PAGE gels have been used to identify a wide range of proteins and such procedures have been summarized in comprehensive reviews by Gabriel and co-workers [22,100–102]. The development of high affinity antibodies to specific proteins has been used quite extensively to identify specific proteins although their in situ use directly in polyacrylamide gels is limited due to the slow diffusion of immunoglobins into the gel matrix [103]. This problem has been circumvented by the development of "Western blotting" [104] procedures for the electrophoretic transfer of separated proteins from polyacrylamide gels to solid membrane support matrixes [e.g., nylon, nitrocellulose, polyvinylidene difluoride (PVDF) membrane] [2–5,105–107]. Membrane-immobilized proteins are then amendable to visualization and detailed analysis using one or a combination of the following techniques: autoradiographic and fluorographic imaging of isotopically labeled proteins, direct dye staining, immunostaining using specific antibodies (immunoblotting), ligand-specific binding, or staining of specific enzymic activities [2,44,103,105,108–110].

4.1. Dye–dye staining

CBB staining combined with silver imaging has been used to intensify [111,112] as well as to differentiate among various classes of proteins [62,113]. Most proteins stain monochromatically with silver yielding predominately brown or black bands although silver stains can produce varying shades of black, blue, brown, red and yellow [19]. Lipoproteins tend to stain blue while some glycoproteins appear yellowish-brown, or red [61]. Color formation is highly dependent upon image development conditions although standardized color-based silver-stain kits are commercially available which reduce staining variability. As reported by Dunbar et al. [114]

post-translational modifications, such as glycosylation or phosphorylation, do not appear to alter polypeptide staining, therefore, p*I* charge trains of modified proteins can be identified on 2D-PAGE gels. The contrasting colors derived from color-based silver stains can also be used to advantage for distinguishing overlapping spots and allows one to detect minor proteins that co-electrophorese with more abundant proteins in overloaded gels that would be indistinguishable using monochromatic stains.

Dzandu et al. [113] have taken advantage of one of the major limitations to the use of silver stain, namely, its inability to stain certain proteins (e.g., various calcium binding proteins such as calmodulin) and combined color silver staining with CBB-R staining to study the human erythrocyte membrane proteins, sialoglycoproteins and lipids. Gels stained first with silver and then with CBB-R showed characteristic blue staining of all CBB-sensitive membrane proteins whereas sialoglycoproteins and lipids were stained yellow with silver stain. Although proteoglycans stain weakly in polyacrylamide gels by traditional stains such as CBB or silver, anionic dyes such as Alcian Blue or toluidine blue are commonly used although the detection sensitivity of these stains is only in the μg range. Moller et al. [115] combined Alcian Blue staining and neutral silver to enhance staining sensitivity 10–100-fold than Alcian Blue combined with diamine silver.

Whereas the visualization of individual proteins using multiple dye staining techniques has been used for specialized purposes, combination dye–dye staining procedures have been replaced by more sensitive radioisotope incorporating methods.

4.2. Double isotope labeling

Double labeling using two different radioisotopes has been described by numerous investigators and has been applied successfully to a wide range of biological problems permitting the simultaneous analysis of two distinct metabolic parameters within the same cell or tissue. Multiple isotopic labeling was originally developed for histological studies and later adapted for use in chromatographic and electrophoretic studies [116–118]. Dual isotopic labeling analysis has found most applications in 2D-PAGE but 1D applications have also been noted. Due to the complexities of 2D-PAGE patterns there are many times where the differences between two patterns is equivocal. Such difficulties can be greatly alleviated, if the two samples to be compared are first radiolabeled with molecules containing different isotopes, the samples mixed and co-electrophoresed on the same 2D-PAGE gel. Dual isotope labeling is based on the selective detection of isotopes of differing energy emission spectra (e.g., ^{14}C and ^{35}S versus ^{3}H, ^{32}P versus ^{35}S, β versus γ emitters) and is most amendable to use where metabolic labeling of protein samples is feasible.

McConkey [119] has utilized double isotope labeling in the analysis of protein expression in *Escherichia coli* and HeLa (human cervical carcinoma) cells. Separate cultures of *E. coli* and HeLa cells were metabolically labeled with [^{14}C]- and [^{3}H]lysine, respectively, the solubilized samples mixed at a dpm ratio of 8 to 1 to adjust for differences in the fluorographic sensitivity of ^{3}H versus ^{14}C [43,94] and co-electrophoresed. Prior to fixation, the 2D-gel was impregnated with PPO, dried, and analyzed on XR5 film using fluorography to detect both ^{14}C-labeled *E. coli* and ^{3}H-labeled HeLa cell proteins, respectively, as illustrated in Fig. 5A and the contact negative (Fig. 5C). The 2D-gel was then exposed to Kodak No-Screen film which is insensitive to light (photons) produced by ^{3}H interaction with PPO and detects only the ^{14}C-labeled *E. coli* proteins by direct autoradiography as shown in Fig. 5B. Comparison of Fig. 5B versus C allows one to identify proteins labeled with either or both isotopes and to identify HeLa cell-specific (white spots in Fig. 5D) and *E. coli*-specific proteins.

In a related procedure described by Walton et al. [117], two distinct populations of human fibroblast cells were labeled with [^{3}H]lysine and [^{14}C]lysine, respectively, mixed to give a ^{3}H/^{14}C ratio of 150:1, and separated by 2D-PAGE. The gel was first analyzed using fluorography to detect emissions from both ^{14}C and ^{3}H. The gel

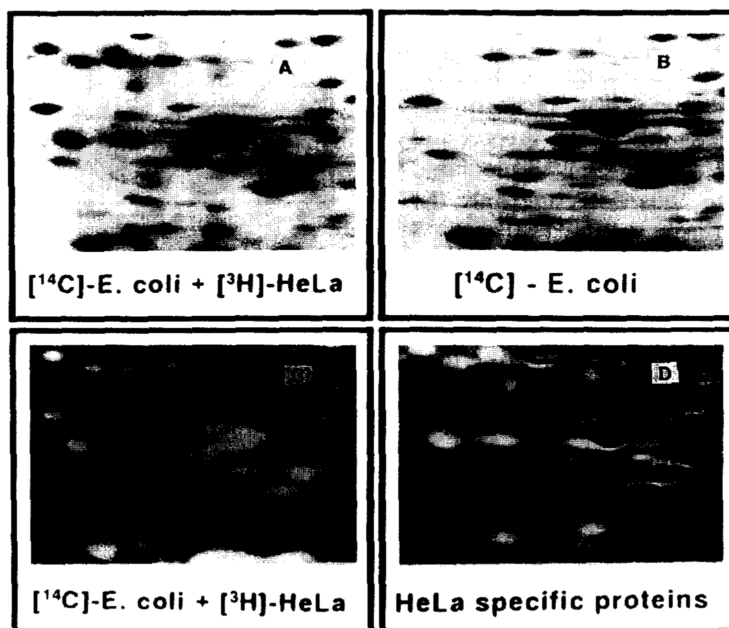

Fig. 5. Comparison of ^{14}C-labeled whole cell extracts from *E. coli* and ^{3}H-labeled whole cell extracts from HeLa cells. (A) Fluorograph of combined ^{14}C-labeled *E. coli* and ^{3}H-labeled HeLa proteins; (B) autoradiograph depicting only ^{14}C-labeled *E. coli* proteins; (C) contact negative (Fine Grain Positive Film) of fluorograph (A); (D) overlay composite of (B) and (C) illustrating (white spots) proteins found in HeLa cells but not in *E. coli* cells. Adapted from [119].

was then painted black with marking ink to screen photons emitted by the ^{3}H and a second exposure was made using autoradiography at room temperature on fresh film revealing ^{14}C-labeled proteins. Cooper and Burgess [120] modified this procedure for the simultaneous detection of either ^{35}S or ^{14}C and ^{32}P. Their procedure utilized aluminum foil and filter paper to provide discrimination between the lower energy β-emitting isotopes (^{35}S and ^{14}C) from the higher-energy (^{32}P). The electropherogram of mixed samples was sandwiched between two X-ray films and simultaneously exposed at −80°C. The top film recorded directly the β-emissions from ^{35}S directly whereas the bottom film (shielded from the autoradiogram by 0.015 mm thick aluminum foil) recorded scintillation photons from an intensifying screen excited by ^{32}P emissions. At room temperature there was considerable spillover exposure of the ^{35}S film by ^{32}P; however, when autoradiography was performed at −80°C the isotopic discrimination was virtually complete.

Harrington et al. [121] and Guy et al. [122] using storage phosphor imaging studied nerve growth factor mediated protein phosphorylation in PC12 rodents cells labeled simultaneously with [^{35}S]methionine and [^{32}P]orthophosphate. Total radioactivity incorporated into protein (including both ^{35}S and ^{32}P) was first imaged by direct exposure of dried 2D-PAGE gel containing ^{32}P- and ^{35}S-labeled proteins to a storage phosphor screen. A second image was then prepared in which a copper foil filter was placed between the gel and the phosphor screen thereby blocking nearly all of the lower energy β emission from the ^{35}S-labeled proteins. Thus the first autoradiograph identified all ^{32}P- and ^{35}S-labeled proteins and the second autoradiogram identified those labeled primarily with ^{32}P. The sensitivity was more than 10-fold greater than that of autoradiogrpahy and required exposures of 24–48 h instead of weeks [121].

Lecocq et al. [123] utilized a combination of β- (^{35}S) and γ- (^{75}Se) emitting isotopes incorporated into methionine analogues for the analysis

of polypeptide expression in human amniotic and V79 cells. Fluorography was used first to detect both ^{35}S and ^{75}Se, while a second autoradiographic exposure was made with a piece of exposed X-ray film interposed between the gel and fresh unexposed film to block ^{35}S-mediated photon and β-emissions thus detecting only the γ-emissions of ^{75}Se. Easty et al. [124] used a similar approach to determine keratinocyte and fibroblast specific protein expression using [^{35}S]methionine and [^{75}Se]selenomethionine-labeled human skin cells. Aluminum foil (0.15 mm) was used to block lower-energy β-emissions from the ^{35}S thus selectively allowing γ-emissions from [^{75}Se]selenomethionine to penetrate to the second film.

We have utilized consecutive silver staining in combination with autoradiography of ^{35}S- and ^{32}P-labeled cellular proteins to simultaneously assess in vitro synthesis and/or degradation as well as the phosphorylation/dephosphorylation of individual cellular polypeptides using a "triple labeling" technique in the in vitro analysis of transforming growth factor type β_1 (TGF-β_1) mediated signal transduction in culture rat liver epithelial (RLE) cells [125]. Following co-electrophoresis of mixed [^{35}S]methionine- and [^{32}P]orthophosphate-labeled RLE cell populations, the 2D-PAGE gels were silver stained [60] and duplicate images of the wet gels made using Kodak X-Omat duplicating film [126,127]. The silver-stained gels were dried and exposed simultaneously to two sheets of Kodak SB-5 X-ray film placed back to back at room temperature. The first film which was placed in direct contact with the dried silver-stained gel visualizes both exposure to ^{35}S and ^{32}P while the second film recorded only exposure to ^{32}P (β-particle from ^{35}S unable to pass through film emulsion and Mylar backing of film). A section from a representative silver-stained image of whole cell RLE lysate polypeptides along with the corresponding autoradiograms of ^{32}P- and mixed $^{32}P/^{35}S$-labeled samples obtained from the dried silver-stained gel is shown in Fig. 6. As illustrated in Fig. 6 an excellent correspondence between the silver-stained gel image (reflective of constitutive levels of polypeptide expression) and the

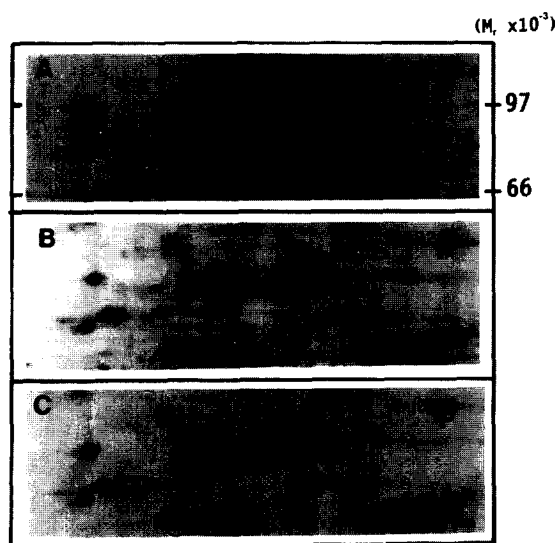

Fig. 6. Simultaneous comparison of silver-stained, [^{35}S]methionine- and [^{32}P]orthophosphate-labeled RLE cellular polypeptides. Panels (A), (B) and (C) represent selected regions between M_r 66 000 and 97 000 and pI 5 and 6. (A) silver-stained polypeptides; (B) ^{35}S- plus ^{32}P-labeled polypeptides; (C) ^{32}P-labeled. Numbered polypeptides indicate representative polypeptides which exhibit varying degrees of expression dependent upon detection technique (e.g., silver staining versus metabolic labeling) [125]. Adapted from [125].

metabolically labeled ^{32}P- and $^{35}S + {}^{32}P$-labeled images (measure of biosynthesis/degradation and phosphorylation status) is obtained. Whereas silver staining presents serious quenching problems for both the autoradiographic and fluorographic detection of ^{3}H-labeled proteins, only minor quenching effects ($<10\%$) are observed using higher energy emitters, such as [^{35}S]methionine or [^{14}C]amino acids as illustrated in Fig. 7 [125]. The juxtapositioning of the silver-stained images with the images obtained from the autoradiographic film permits the unambiguous mapping of the phosphorylated polypeptides back to their corresponding silver-stained and [^{35}S]methionine-labeled counterparts. This technique makes it possible to analyze and measure both the constitutive levels of protein expression (silver staining) with rates of protein synthesis and/or degradation (metabolic labeling with [^{35}S]methionine) and protein phos-

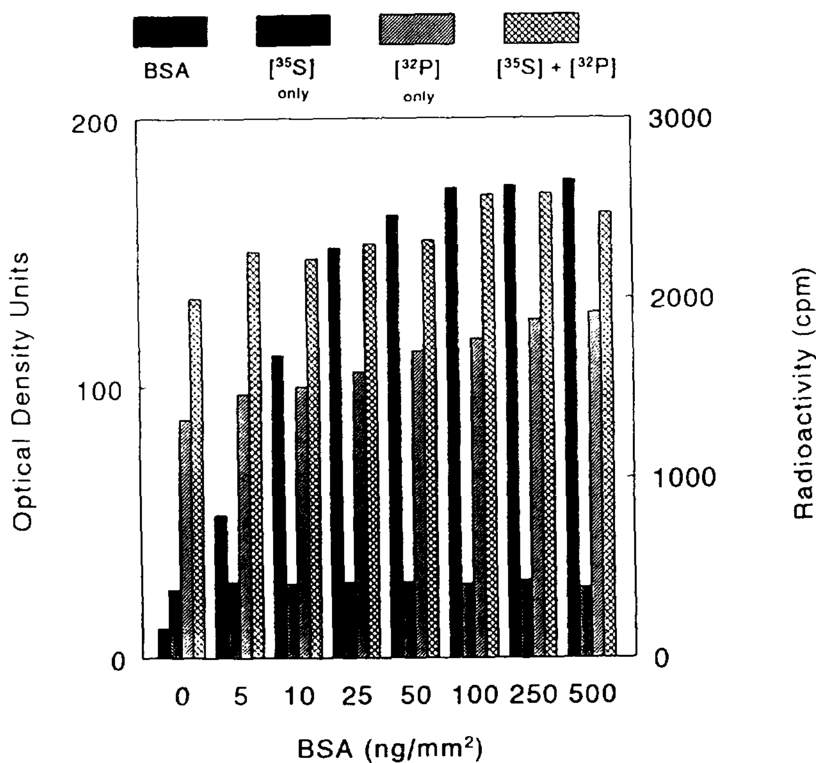

Fig. 7. Effect of silver staining on autoradiographic detection of ^{35}S- and ^{32}P-labeled polypeptides. A series of individual polyacrylamide gel strips were cast containing increasing concentrations of unlabeled bovine serum albumin (0–500 ng/mm^2) along with constant concentrations of either [^{35}S]methionine or [^{32}P]orthophosphate-labeled cellular lysates as well as mixed ^{35}S plus ^{32}P samples. Gel strips were silver-stained, duplicate images made using Kodak X-OMAT duplicating film, and dried gels subjected to autoradiography. Electrophoretograms (duplicate images and autoradiograms were analyzed using the Elsie 5 computer analysis system developed for aiding in the digitalization, segmentation, quantitation, interimage comparison and analysis of 2D-PAGE images [165,166].

phorylation status ([^{32}P]orthophosphate labeling).

Whereas the majority of the studies incorporating dual isotope analysis have been performed in in vitro systems, Kuhn et al. [128] have combined conventional fluorography of 2D-PAGE gel patterns of in vivo [^{35}S]methionine-labeled mouse serum and whole tissue proteins to obtain a "biosynthetic image" followed by rehydration of the gel and subsequent silver staining to generate a "constitutive image" of separated proteins. The major advantage of Kuhn et al.'s procedure is that it permits the analysis of the biosynthesis of proteins in situ, under normal physiological conditions. Attempts to increase sensitivity by initial silver staining followed by fluorography of the dried silver-stained gels were not successful probably due to silver quenching of the scintillant-mediated fluorographic enhancement [42,43].

4.3. Immunological analysis

Western immunoblot analysis of separated proteins represents one of the most powerful analytical tools available for the characterization of proteins. The development of high-affinity antibodies to specific proteins has been used quite extensively to identify specific proteins although their use directly in polyacrylamide gels is limited due to the slow diffusion of immunoglobins into the gel matrix. This problem has

been circumvented by the development of "Western blotting" [2,104] procedures for electrophoretic the transfer of separated proteins from polyacrylamide gels to solid membrane support matrixes (e.g., nylon, nitrocellulose, PVDF membrane) [2–5,106,107]. Sensitive staining of total protein patterns transferred to nitrocellulose membranes can be achieved with most of the commonly used dyes or stains including Amido Black [129,130], Fast green FCF [131], CBB [104,130], India ink [132,133], Ponceau S [134–136], silver [111], iron [132,137] and colloidal gold [138,139]. Christiansen and Houen [140] have comprehensively compared commonly used staining procedures for PVDF membranes including CBB, mercurochrome, silver, colloidal gold and dimethylaminoazobenzene isothiocyanate. CBB was the most versatile being completely compatible with subsequent biochemical procedures including N-terminal Edman degradation amino acid microsequencing and immunostaining. Gold and silver were the most sensitive while mercurochrome allowed for the detection of sulfhydryl-containing proteins. Bickar and Reid [141] have recently described a high-affinity non-specific protein stain applicable for use on nitrocellulose membranes, tissue prints as well as polyacrylamide and agarose gels using copper phthalocyanine 3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt (CPTS). CPTS staining is rapid (<1 min), reversible with pH change, and more sensitive than any of the previously reported organic dye-based staining methods (10 ng CPTS versus 20 ng Fast green and 1 ng gold) [131,141,142].

Individual proteins can be characterized by their capacity to react either with specific antibodies or group specific ligands (e.g., lectins, DNA, RNA or metals). The primary antibodies can be labeled for direct observation but indirect sandwich techniques using either a second antibody or *Staphylococcus aureus* protein A or protein G which have been conjugated with detectable label(s), are significantly more sensitive. A diverse range of labels and detection systems have been described in the literature. The second antibody can be radiolabeled (^{125}I) [104,143], conjugated to various enzymes (e.g, horseradish peroxidase, alkaline phosphatase, β-galatosidase) [144,145], complexed to biotin/streptavidin (or avidin) [146], heavy metals (e.g, gold/silver) [138,147] or fluorescently labeled (fluorescein isothiocyanate) [3,148]. Detection methods have involved enzyme histochemistry using chromogenic substrates, inorganic colorimetric reactions, and autoradiography. Recently, a non-radioactive chemiluminescence (CL)-based protocol for the detection of immobilized antigens (proteins), conjugated either directly or indirectly with horseradish peroxidase-labeled antibodies, has been commercially developed (Amersham) [149]. The technique is extremely sensitive, capable of detecting less than 1 pg of protein, and allows for rapid and multiple stripping/reprobing of blots with no antigen damage. A schematic representation of these procedures is illustrated in Fig. 8.

Multiple immunoblotting and staining techniques have been utilized to detect multiple antigens on a single electropherogram [150]. Initial attempts to stain polyacrylamide gels, photograph the images, followed by destaining, and Western transfer for immunodetection have been described. These methods, however, are less than optimal since repeated staining and destaining of gels results in significant fixation of proteins with the polyacrylamide gel matrixes thus significantly decreasing proteins electrotransfer efficiencies. Wise and Lin [151] have described methods for the high-efficiency electrotransfer of silver-stained proteins from polyacrylamide gels to PVDF membranes (Immobilon-P). The efficiency of transfer ranged from 85–100% with complete retention of antigenicity of the transferred proteins [151].

Kaufmann et al. [150] have developed "erasable" Western blotting procedures for the efficient removal of primary and secondary antibodies from nitrocellulose and nylon membranes with no loss of immobilized polypeptides from original blots. This has permitted repeated probing of single blots with multiple antibodies. Double staining of immunoblot membranes using enzyme histochemistry and organic dyes, such as CBB, Ponceau S and India ink have proved extremely useful for the unambiguous

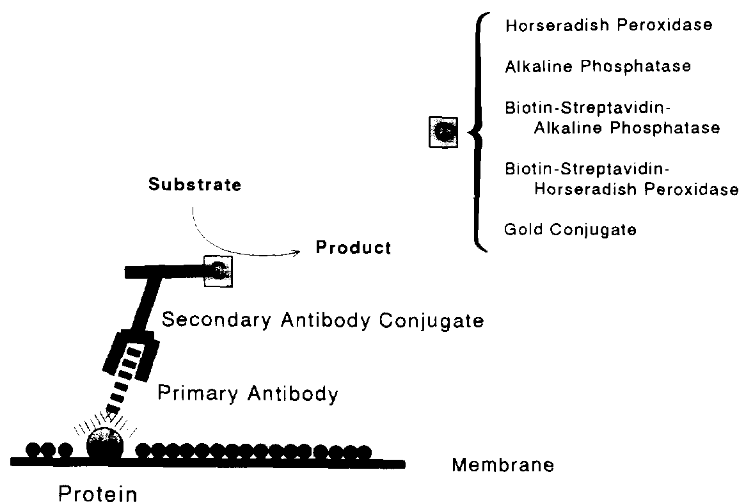

Fig. 8. Schematic illustration of immunoblot detection of membrane bound proteins (antigens). Gelatin, BSA or non-fat dry milk block unoccupied protein binding sites on the membrane. Primary antibody to a specific protein (antigen) is incubated with the membrane. A second antibody–enzyme conjugate is added to bind to the primary antibody. Color development reagent is utilized to localize antigen–antibody–antibody complex [substrate→product].

identification of specific proteins on both 1D- and 2D-PAGE electropherograms. Steffen and Linck have utilized a sequential combination of horseradish peroxidase/luminescence, alkaline phosphatase and silver-enhanced immunogold staining to detect three different antigens on a single 2D-Western blot without eluting the antibody–dye complex between the staining of single polypeptides [152]. Ono and Tuan [153] used immunoblot analysis first to identify the vitamin D-dependent calcium binding protein calbindin-D_{28K} followed by counterstaining with India ink to visualize total protein expression.

We and others have combined immunoprecipitation to enrich for a particular protein population, namely phosphotyrosine containing proteins, in whole cell lysate preparations followed by 2D-PAGE separation and Western immunoblot analysis. Blotted membranes were initially stained with colloidal gold to identify total phosphotyrosine proteins followed by treatment with primary antibody directed against the specific proteins such as the epidermal growth factor receptor (EGF-R) and [^{125}I]protein A to localize the EGF-R as illustrated in Fig. 9.

Glycoproteins have also been detected immunologically using "ligand overlaying" on Western blots [154,155]. Glycoproteins can conveniently be visualized on nitrocellulose blots with radiolabeled [156] lectin (concanavalin A) overlays or peroxidase conjugated [157] lectins. Immobilized glycoproteins can be chemically modified at sugar moieties generating aldehyde groups which can be detected with an enzyme–hydrazide complex [155] Lipopolysaccharides (LPSs) have also been detected immunochemically following Western transfer [158]. Immobilized LPSs were immunoautoradiographically visualized with anti-LPS antibody and subsequent treatment with [^{125}I]protein A.

Other types of protein–ligand binding interactions can similarly be detected following Western blotting using combination dye staining, radioactive labeling and antibody detection. These include protein–DNA ("Southwestern" blotting) [159,160], protein–metal [84,161,162], protein–hormone receptor [163] and protein–protein interactions [164].

5. Summary and concluding remarks

Polyacrylamide gel electrophoresis, in particular 2D-PAGE, remains the method of choice for

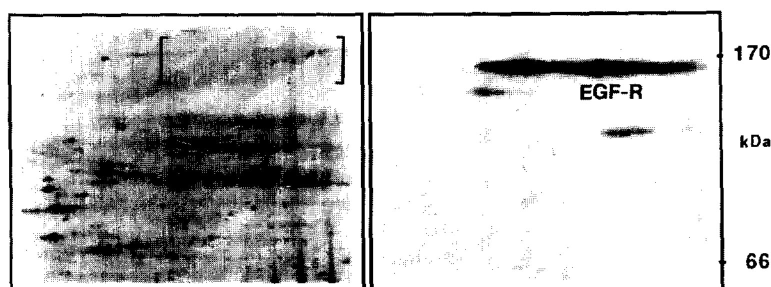

Fig. 9. Western immunoblot analysis of EGF-R. NIH-3T3 (clone-56) cells that had been previously transfected with human EGF-R to get 10^6 receptors/cell were treated with EGF and total tyrosine phosphorylated polypeptides immunoprecipitated from cellular lysates with anti-phosphotyrosine antibodies. The immunoprecipitates were separated using 2D-PAGE, transferred to Immobilon membranes and total phosphotyrosine polypeptides stained with colloidal gold (left). The stained membrane was then incubated with anti-EGF-R antibody followed by [^{125}I]protein A and exposed to Kodak XAR X-ray film (intensifying screen) for three days at −70°C (right). kDa = kilodalton.

the analysis of complex protein mixtures capable of resolving upward to 2000 individual polypeptides on a single electropherogram. This has necessitated the development of highly sensitive and selective protein-visualization protocols incorporating both non-radioactive dye staining and radioisotopic imaging methodologies. The combined use of immunodetection using high-affinity antibodies to specific proteins with dye staining and radiological detection has permitted the detection and analysis of individual cellular polypeptides some of which may be expressed as single copy gene products. There is no doubt that PAGE will play a crucial role in the separation and analysis of cellular polypeptides in both clinical and basic biochemical research. As such the future development and utilization of highly selective and sensitive protein detection protocols is paramount.

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References

[1] P.H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
[2] U. Beisiegel, *Electrophoresis*, 7 (1986) 1.
[3] H. Towbin, T. Staehelin and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4350.
[4] J.M. Gershoni and G.E. Palade, *Anal. Biochem.*, 131 (1983) 1.
[5] G. Bers and G. Garfin, *Biotechniques*, 3 (1985) 276.
[6] I.P. Griffith, *Anal. Biochem.*, 46 (1972) 402.
[7] A. Datyner and E.D. Finnimore, *Anal. Biochem.*, 55 (1973) 479.
[8] C.M. Wilson, *Methods Enzymol.*, 91 (1983) 236.
[9] R.C. Switzer III, C.R. Merril and S. Shifrin, *Anal. Biochem.*, 98 (1979) 231.
[10] C.R. Merril, R.C. Switzer III and M.L. Van Keuren, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4335.
[11] C.R. Merril, D. Goldman and M.L. Van Keuren, *Electrophoresis*, 3 (1982) 17.
[12] C.R. Merril, D. Goldman and M.L. Van Keuren, *Methods Enzymol.*, 96 (1983) 230.
[13] C.R. Merril and M.G. Harrington, *Clin. Chem.*, 30 (1984) 1938.
[14] D.F. Hochstrasser, M.G. Harrington, A.-K. Hochstrasser, M.J. Miller and C.R. Merril, *Anal. Biochem.*, 173 (1988) 424.
[15] C.R. Merril, *Methods Enzymol.*, 182 (1990) 477.
[16] B.R. Oakley, D.R. Kirsch and N.R. Morris, *Anal. Biochem.*, 105 (1980) 361.
[17] W. Wray, T. Boulikas, V.P. Wray and R. Hancock, *Anal. Biochem.*, 118 (1981) 197.
[18] J.H. Morrissey, *Anal. Biochem.*, 117 (1981) 307.
[19] D.W. Sammons, L.D. Adams and E.E. Nishizawa, *Electrophoresis*, 2 (1981) 135.
[20] B.D. Hames and D. Rickwood, in B.D. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: A Practical Approach*, IRL Press, New York, 2nd ed., 1990, p. 324.
[21] L.M. Leong, B.H. Tan and K.K. Ho, *Anal. Biochem.*, 207 (1992) 317.
[22] O. Gabriel and D.M. Gersten, *Anal. Biochem.*, 203 (1992) 1.

[23] J.E. Gander, *Methods Enzymol.*, 104 (1984) 447.
[24] J.A. Cutting, *Methods Enzymol.*, 104 (1984) 451.
[25] J.P. Prat, J.N. Lamy and J.D. Weill, *Bull. Soc. Chim. Biol.*, 51 (1969) 1367.
[26] L.E. King and M. Morrison, *Anal. Biochem.*, 71 (1976) 223.
[27] A.E. Dahlberg, C.W. Dingman and A.C. Peacock, *J. Mol. Biol.*, 41 (1969) 139.
[28] R. McMaster-Kaye and J.S. Kayes, *Anal. Biochem.*, 61 (1974) 120.
[29] L.E. Gander, *Methods Enzymol.*, 104 (1984) 447.
[30] J.A. Cutting, *Methods Enzymol.*, 104 (1985) 451.
[31] H.J. Moller, D. Heingard and J.H. Poulsen, *Anal. Biochem.*, 209 (1993) 169.
[32] G. Weber, G. Schwamberger and E. Ferber, *Anal. Biochem.*, 209 (1993) 251.
[33] C. Queiroz-Claret and J.-C. Meunier, *Anal. Biochem.*, 209 (1993) 228.
[34] H. Min and M.K. Cowman, *Anal. Biochem.*, 155 (1986) 275.
[35] K.P. Campbell, D.H. MacLennan and A.O. Jorgensen, *J. Biol. Chem.*, 258 (1983) 11267.
[36] R.A. Wallace and P.C. Beqovac, *J. Biol. Chem.*, 260 (1985) 11268.
[37] A. Al-Hakim and R.J. Linhardt, *Electrophoresis*, 11 (1990) 23.
[38] R.M. Zacharius, T.E. Zell, J.H. Morrison and J.J. Woodlock, *Anal. Biochem.*, 30 (1969) 148.
[39] M.R. Green and J.V. Paslewka, *Anal. Biochem.*, 65 (1975) 66.
[40] R.A. Wallace and J.P. Morgan, *Anal. Biochem.*, 157 (1986) 256.
[41] E.J. Hahn, *Am. Lab.*, 15 (1983) 64.
[42] W.M. Bonner and R.A. Laskey, *Eur. J. Biochem.*, 46 (1974) 83.
[43] R.A. Laskey and A.D. Mills, *Eur. J. Biochem.*, 56 (1975) 335.
[44] A.T. Andrews, *Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications*, Oxford Univ. Press, Oxford, 2nd ed., 1986.
[45] M.J. Dunn, *J. Chromatogr.*, 418 (1987) 145.
[46] B.S. Dunbar, in B.S. Dunbar (Editor), *Two-Dimensional Electrophoresis and Immunological Techniques*, Plenum Press, New York, 1987, p. 67.
[47] F. Fazekas de St. Groth, R.G. Webster and A. Datyner, *Biochim. Biophys. Acta*, 71 (1963) 377.
[48] P.G. Righetti, in T.S. Work and E. Work (Editors), *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 11, North-Holland, Amsterdam, 1983, p. 148.
[49] V. Neuhoff, R. Stamm and H. Eibl, *Electrophoresis*, 6 (1985) 427.
[50] B.D. Hames, in B.D. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: A Practical Approach*, IRL Press, New York, 2nd ed., 1990, p. 321.
[51] A.H. Reisner, P. Nemes and C. Bucholtz, *Anal. Biochem.*, 64 (1975) 509.
[52] C.M. Wilson, *Anal. Biochem.*, 96 (1979) 263.
[53] M.A. Gorovsky, K. Carlson and J.L. Rosenbaum, *Anal. Biochem.*, 35 (1970) 359.
[54] R.E. Allen, K.C. Masak and P.K. McAllister, *Anal. Biochem.*, 104 (1980) 494.
[55] H.-Y. Hong, J.-K. Choi and G.-S. Yoo, *Anal. Biochem.*, 214 (1993) 96.
[56] W.J.H. Nauta and P.A. Gygax, *Stain Technol.*, 26 (1951) 5.
[57] M.G. Harrington and C.R. Merril, *Clin. Chem.*, (1984) 1933.
[58] C.R. Merril, M. Harrington and V. Alley, *Electrophoresis*, 5 (1985) 289.
[59] G. Berson, *Anal. Biochem.*, 134 (1983) 230.
[60] D.F. Hochstrasser, A. Patchornik and C.R. Merril, *Anal. Biochem.*, 173 (1988) 412.
[61] D. Goldman, C.R. Merril and M.H. Ebert, *Clin. Chem.*, 26 (1980) 1317.
[62] C. Rochette-Egly and C. Stussi-Garaud, *Electrophoresis*, 5 (1984) 285.
[63] M. Schleicher and D.M. Watterson, *Anal. Biochem.*, 131 (1983) 312.
[64] B.O. Barger, F.C. White, J.L. Pace, D.L. Kemper and W.L. Ragland, *Anal. Biochem.*, 70 (1976) 327.
[65] J.L. Pace, D.L. Kemper and W.L. Ragland, *Biochem. Biophys. Res. Commun.*, 57 (1974) 482.
[66] H. Schetters and B. McLeod, *Anal. Biochem.*, 98 (1979) 329.
[67] R.E. Stephens, *Anal. Biochem.*, 65 (1975) 369.
[68] P. Tjissen and E. Kurstak, *Anal. Biochem.*, 99 (1979) 97.
[69] P.R. Eng and C.O. Parker, *Anal. Biochem.*, 59 (1974) 323.
[70] K. Yamamoto, Y. Okamoto and T. Sekine, *Anal. Biochem.*, 84 (1978) 313.
[71] E. Weidekamm, D.F.H. Wallach and R. Fluckiger, *Anal. Biochem.*, 54 (1973) 102.
[72] W.L. Ragland, J.L. Pace and D.L. Kemper, *Anal. Biochem.*, 59 (1974) 24.
[73] B.K. Hartman and S. Udenfriend, *Anal. Biochem.*, 30 (1969) 391.
[74] P.M. Harowitz and S. Bowman, *Anal. Biochem.*, 165 (1987) 430.
[75] G. Jackowski and C.C. Liew, *Anal. Biochem.*, 102 (1980) 321.
[76] S.D. Carson, *Anal. Biochem.*, 78 (1977) 428.
[77] J.K. Dzandu, J.F. Johnson and G.E. Wise, *Anal. Biochem.*, 174 (1988) 157.
[78] C. Lee, A. Lewin and D. Branton, *Anal. Biochem.*, 166 (1987) 308.
[79] L.D. Adams and K.M. Weaver, *Appl. Theor. Electrophoresis*, 1 (1990) 279.
[80] M.L. Ortiz, M. Calero, C.F. Patron, L. Castellanos and E. Mendez, *FEBS Lett.*, 296 (1992) 300.
[81] C. Fernandez-Patron, L. Castellanos-Serra and P. Rodriquez, *Biotechniques*, 12 (1992) 564.
[82] M. Ferreras, J.G. Gavilanes and J.M. Garcia-Segura, *Anal. Biochem.*, 213 (1993) 206.

[83] Y. Chen and J. Drysdale, *Anal. Biochem.*, 212 (1993) 47.
[84] K. Maruyama, T. Mikawa and S. Ebashi, *J. Biochem. (Tokyo)*, 95 (1984) 511.
[85] S.-M. Lin, S.M. Hopfer, S.M. Brennan and F.W. Sunderman, *Res. Commun. Chem. Pathol. Pharmacol.*, 65 (1989) 275.
[86] J.I. Garrels and B.R.J. Franza, *J. Biol. Chem.*, 264 (1989) 5283.
[87] B.S. Dunbar, in B.S. Dunbar (Editor), *Two-Dimensional Electrophoresis and Immunological Techniques*, Plenum Press, New York, 1987, p. 103.
[88] C.W. Parker, *Meth. Enzy.*, 182 (1990) 721.
[89] P.J. Wirth, J. Doniger, S.S. Thorgeirsson and J.A. DiPaolo, *Cancer Res.*, 46 (1986) 390.
[90] P.J. Wirth, S.H. Yuspa, S.S. Thorgeirsson and H. Hennings, *Cancer Res.*, 47 (1987) 2831.
[91] D. Rickwood, in B.D. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: A Practical Approach*, IRL Press, New York, 2nd ed., 1990, p. 346.
[92] E.J. Zapolski, D.M. Gersten and R.S. Ledley, *Anal. Biochem.*, 123 (1982) 325.
[93] G. Graham, R.S. Nairn and G.W. Bates, *Anal. Biochem.*, 88 (1978) 434.
[94] R.A. Laskey, *Methods Enzymol.*, 65 (1980) 363.
[95] R.F. Johnston, S.C. Pickett and D.L. Barker, *Methods: A Companion to Methods Enzymol.*, 3 (1991) 128.
[96] R.F. Johnston, S.C. Pickett and D.L. Barker, *Electrophoresis*, 11 (1990) 355.
[97] R. Swanstrom and P.R. Shank, *Anal. Biochem.*, 86 (1978) 184.
[98] J. Bossinger, M.J. Miller, K.-P. Vo, E.P. Geiduschek and N.-H. Xuong, *J. Biol. Chem.*, 254 (1979) 7986.
[99] Y. Amemiya and J. Miyahara, *Nature*, 336 (1988) 89.
[100] O. Gabriel, in S.P. Colowick and N.O. Kaplan (Editors), *Methods Enzymol.*, 22 (1971) 578.
[101] M.J. Heeb and O. Gabriel, *Methods Enzymol.*, 104 (1984) 416.
[102] D.M. Gersten and O. Gabriel, *Anal. Biochem.*, 203 (1992) 181.
[103] B.S. Dunbar (Editor), *Two-Dimensional Electrophoresis and Immunological Techniques*, Plenum Press, New York, 1987.
[104] W.N. Burnette, *Anal. Biochem.*, 112 (1981) 195.
[105] J.M. Gershoni, *Methods Biochem. Anal.*, 33 (1988) 1.
[106] H. Towbin, T. Staehelin and J. Gordon, *J. Clin. Chem. Clin. Biochem.*, 27 (1989) 495.
[107] J. Renart, J. Reiser and G.R. Stark, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 3116.
[108] T.M. Timmons and B.S. Dunbar, *Methods Enzymol.*, 182 (1990) 679.
[109] J.M. Gershoni, *Trends Biochem. Sci.*, 10 (1985) 103.
[110] B.D. Hames, in B.D. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: A Practical Approach*, IRL Press, New York, 2nd ed., 1990, p. 1.
[111] M.R. DeMoreno, J.F. Smith and R.V. Smith, *Anal. Biochem.*, 151 (1985) 466.
[112] M. Ross and L. Peters, *Biotechniques*, 9 (1990) 532.
[113] J.K. Dzandu, M.E. Deh, D.L. Barratt and G.E. Wisw, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 1733.
[114] B.S. Dunbar, A.B. Dudkiewicz and D.S. Bundman, *Biol. Reprod.*, 32 (1985) 619.
[115] H.J. Moller, D. Heinegard and J.H. Poulsen, *Anal. Biochem.*, 209 (1993) 169.
[116] R.L. Trelstad, *Exper. Cell Res.*, 39 (1965) 318.
[117] K.E. Walton, D. Stryer and E.I. Gruenstein, *J. Biol. Chem.*, 254 (1979) 7951.
[118] E.I. Gruenstein and T.W. Smith, *Anal. Biochem.*, 61 (1974) 429.
[119] E.H. McConkey, *Anal. Biochem.*, 96 (1979) 39.
[120] P.C. Cooper and A.W. Burgess, *Anal. Biochem.*, 126 (1982) 301.
[121] M.G. Harrington, L. Hood and C. Puckett, *Methods: A Companion to Methods Enzymol.*, 3 (1991) 135.
[122] G.R. Guy, R. Philip and Y.H. Tan, *Electrophoresis*, 15 (1994) 417.
[123] R. Lecocq, A. Hepburn and F. Lamy, *Anal. Biochem.*, 127 (1982) 292.
[124] D.J. Easty, K. Patel, R. Dover, D.J. Evans and M.J. Dunn, *Electrophoresis*, 9 (1988) 227.
[125] L. Luo and P.J. Wirth, *Electrophoresis*, 14 (1993) 127.
[126] P.J. Wirth, T. Benjamin, D.M. Schwartz and S.S. Thorgeirsson, *Cancer Res.*, 46 (1986) 400.
[127] P.J. Wirth, M.S. Rao and R.P. Evarts, *Cancer Res.*, 47 (1987) 2839.
[128] L. Kuhn, J. Kettman and I. Lefkovits, *Electrophoresis*, 10 (1989) 708.
[129] W. Schaffner and C. Weissmann, *Anal. Biochem.*, 56 (1973) 502.
[130] W. Lin and H. Kasamatsu, *Anal. Biochem.*, 128 (1983) 302.
[131] M. Reinhart and D. Malamud, *Anal. Biochem.*, 123 (1982) 229.
[132] K.W. Li, W.P.M. Geraerts, R. van Elk and J. Joose, *Anal. Biochem.*, 182 (1989) 44.
[133] K. Hancock and V.C.W. Tsang, *Anal. Biochem.*, 133 (1983) 157.
[134] R. Montelaro, *Electrophoresis*, 8 (1987) 432.
[135] R.H. Aebersold, J. Leavitt, R.A. Saavedra, L.E. Hood and S.B.H. Kent, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 6970.
[136] O. Salinovich and R.C. Montelaro, *Anal. Biochem.*, 156 (1986) 341.
[137] M. Moeremans, M. DeRaeymaeker, G. Daneels and J. DeMey, *Anal. Biochem.*, 153 (1986) 18.
[138] Y.-H. Hsu, *Anal. Biochem.*, 142 (1984) 221.
[139] M. Moeremans, G. Daneels and J. DeMay, *Anal. Biochem.*, 145 (1985) 315.
[140] J. Christiansen and G. Houen, *Electrophoresis*, 13 (1992) 179.
[141] D. Bickar and P.D. Reid, *Anal. Biochem.*, 203 (1992) 109.
[142] K. Li, P. Geraerts, R. van Elk and J. Joosse, *Anal. Biochem.*, 182 (1989) 44.
[143] J. Renart and I.V. Sandoval, *Methods Enzymol.*, 104 (1984) 455.

[144] V.C.W. Tsang, J.M. Peralta and A.R. Simons, *Methods Enzymol.*, 92 (1983) 377.
[145] M.S. Blake, K.H. Johnston, G.J. Russell-Jones and E.C. Gotschlich, *Anal. Biochem.*, 136 (1984) 175.
[146] E. Harlow and D. Lane, in E. Harlow and D. Lane (Editors), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, p. 319.
[147] M. Moeremans, G. Daneels, A. Van Dijck, G. Langanger and J. DeMey, *J. Immunol. Methods*, 74 (1983) 353.
[148] T.H. The and T.E.W. Feltkemp, *Immunology*, 18 (1970) 875.
[149] T.P. Whitehead, L.J. Kricka, T.J. Carter and G.H. Thorpe, *Clin. Chem.*, 25 (1979) 1531.
[150] S.H. Kaufmann, C.M. Ewing and J.H. Shaper, *Anal. Biochem.*, 161 (1987) 89.
[151] G.E. Wise and F. Lin, *J. Biochem. Biophys. Methods*, 22 (1991) 223.
[152] W. Steffen and R.W. Linck, *Electrophoresis*, 10 (1989) 714.
[153] T. Ono and R.S. Tuan, *Anal. Biochem.*, 187 (1990) 324.
[154] R. Rohringer and D.W. Holden, *Anal. Biochem.*, 144 (1985) 118.
[155] J.M. Gershoni, E.A. Bayer and M. Wilcheck, *Anal. Biochem.*, 146 (1985) 59.
[156] J.M. Gershoni and G.E. Palade, *Anal. Biochem.*, 124 (1982) 396.
[157] M. Moroi and S.M. Jung, *Biochim. Biophys. Acta*, 798 (1984) 295.
[158] W.C. Bradbury, S.D. Mills, M.A. Preston, L.J. Barton and J.L. Penner, *Anal. Biochem.*, 137 (1984) 129.
[159] B. Bowen, J. Steinberg, U.K. Laemmli and H. Weintraub, *Nucl. Acid Res.*, 11 (1980) 1.
[160] S.O. Hoch, *Biochem. Biophy Res. Commun.*, 106 (1982) 1353.
[161] A. Mazen, G. Gradwohl and G. DeMurcia, *Anal. Biochem.*, 172 (1988) 39.
[162] Y. Aoki, M. Kunimoto, Y. Shibata and K. Suzuki, *Anal. Biochem.*, 157 (1986) 117.
[163] J.A. Fernandez-Pol, *FEBS Lett.*, 143 (1982) 86.
[164] M.L. Bell and E. Engvall, *Anal. Biochem.*, 123 (1982) 329.
[165] K.-P. Vo, M.J. Miller, E.P. Geiduschek, C. Nielson, A.D. Olson and N.-H. Xuong, *Anal. Biochem.*, 112 (1981) 258.
[166] A.D. Olson and M.J. Miller, *Anal. Biochem.*, 169 (1988) 49.