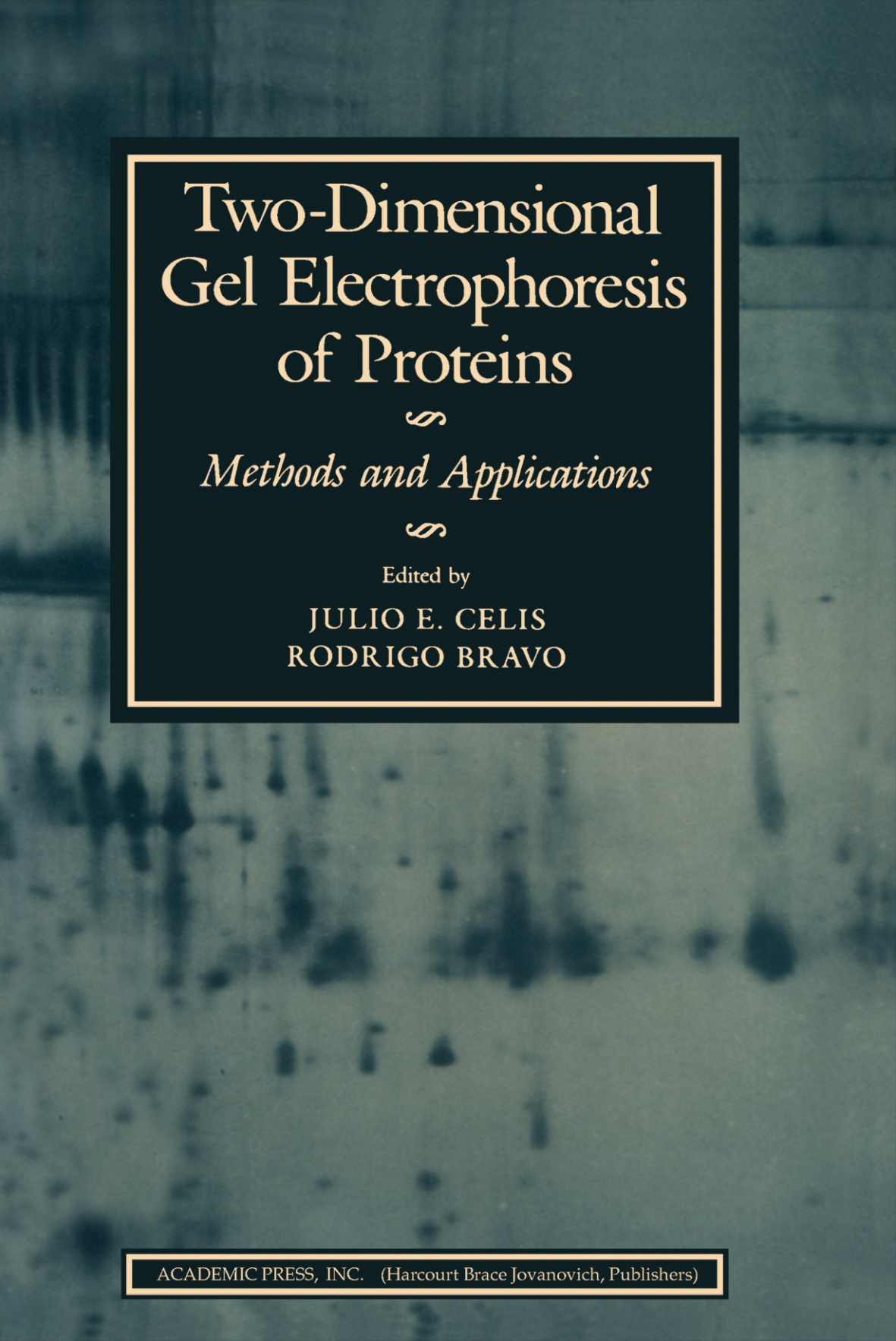

Two-Dimensional Gel Electrophoresis of Proteins

Methods and Applications

Edited by

JULIO E. CELIS
RODRIGO BRAVO

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METHODS AND APPLICATIONS

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METHODS AND APPLICATIONS

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Preface

Eight years have elapsed since O'Farrell first introduced two-dimensional gel electrophoresis as a high-resolution technique for the separation of thousands of proteins from a given cell type. Since then there have been many technological advances that have led to improvements in the methods of detecting, quantifying, comparing, characterizing, and storing information contained in the gels. The technique, however, has not been fully standardized, and various laboratories use slightly different gel running conditions and sample preparation procedures. In most fields in which this technique is being applied there is no consensus as to how gels should be presented (e.g., with respect to the direction of the pH gradient). Despite these minor drawbacks it is timely to publish a volume on two-dimensional gel electrophoresis of proteins, especially because no comprehensive compilation of these techniques or data is available. This book attempts to present an overview of current procedures and to review a few areas of research in which this technique is currently being applied.

The book has been divided into three sections: General Methodology, Applications, and Protein Catalogs. The first chapter in Section I describes the methodology of two-dimensional gel electrophoresis. The following chapters describe computerized two-dimensional gel electrophoresis, silver staining, immunoblotting, and one- and two-dimensional peptide mapping. In most cases, a step-by-step guide to the techniques is given so that procedures may be easily repeated. Section II is dedicated mainly to applications of two-dimensional gel electrophoresis. Because of space limitations, it has not been possible to review all fields in which this technique is currently being applied. Rather we have chosen to reflect current research interests by paying special attention to expanding subjects. These include applications in clinical and cancer research, human genetics, protein biosynthesis, and gene expression in plants. Section III presents current protein catalogs of *Escherichia coli* and human HeLa cells. A catalog of mouse fibroblast proteins is also presented in Section I.

The book is suitable for young researchers as well as for senior scientists working with a wide variety of problems in molecular and cell biology, basic biochemistry, genetics, and clinical research.

We would like to thank the authors for their readiness to write the chapters and for their punctuality in providing manuscripts. Our gratitude is also due to the staff of Academic Press for their aid and cooperation in the completion of this work.

J. E. Celis
R. Bravo

PART I

Methods and Satellite Techniques

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CHAPTER 1

Two-Dimensional Gel Electrophoresis: A Guide for the Beginner

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TWO-DIMENSIONAL GEL ELECTROPHORESIS OF PROTEINS

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I. Introduction

The two-dimensional gel electrophoresis system that gives the best resolution for separating a complex mixture of proteins combines the techniques of isoelectrofocusing (IEF) in the presence of urea and a neutral detergent in the first dimension and slab gel electrophoresis under denaturing conditions using sodium dodecyl sulfate (SDS) in the second dimension (Klose, 1975; O'Farrell, 1975; Scheele, 1975; Iborra and Buhler, 1976). The separation makes use of two independent protein characteristics: one is the charge, which is reflected by the isoelectric point (pI), and the other is the molecular weight, which determines the mobility of the SDS–protein complexes in polyacrylamide gels (Weber and Osborn, 1969).

O'Farrell (1975) first demonstrated the great potential of such a technique when using isotopically labeled proteins by resolving more than a thousand polypeptides and detecting components as minor as 0.001% or less of the total cellular protein. This method has been extensively applied in resolving proteins from both prokaryotic and eukaryotic organisms and can be used for the separation of many types of cellular proteins [for references, see special issue of *Clinical Chemistry* (Vol. 28, No. 4, Part II, pp. 737–1092, 1982) and other chapters in this volume]. Unfortunately, basic proteins are not well separated in the IEF/SDS system, because in general they enter the IEF gel poorly. Even when more basic ampholytes are included, the extension of the pH gradient is very small, because in the presence of urea the basic region of the gradient is unstable. Furthermore, the few basic proteins that enter the IEF gel under these conditions always produce streaks. However, it is possible to overcome these problems using a nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension as described by O'Farrell *et al.* (1977). The main differences between NEPHGE and IEF are that in the former the samples are applied to in the acidic side of the gel and the voltage × time product is smaller than it is in IEF. Under such conditions the pH gradient does not reach full equilibrium. As a result the proteins are not totally focused at their isoelectric point as they are in IEF gels. Nevertheless, most proteins in NEPHGE gels are separated according to their charge differences.

In general, special, not easily attainable apparatus is needed to improve the resolution of the technique, making an initial start difficult for the beginner (Garrels and Gibson, 1976; Garrels, 1979; N. G. Anderson and N. L. Anderson, 1978; N. L. Anderson and N. G. Anderson, 1978). Nevertheless, it is the author's experience that many studies can be done using the standard equipment found in any laboratory. However,

when the highest resolution and fully quantitative analysis are required, then special apparatus is essential.

It is the purpose of this article to describe the IEF–SDS and NEPHGE–SDS techniques using a simple setup and to give the beginner enough basic knowledge for the critical evaluation of the results.

II. Materials and Solutions

A. Materials

Acrylamide, agarose, bisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) were obtained from Bio-Rad. Urea "ultrapure" was obtained from Schwartzman, and Tris-base, Tris-hydrochloride, 2,5-diphenyloxazole (PPO), β-mercaptoethanol, and glycine were obtained from Sigma. Ampholytes were obtained from LKB and Serva. Sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) were from Serva. DNase I and RNase A were from Worthington. Nonionic detergent P40 (NP-40) was obtained from Fluka. Dimethyl sulfoxide (DMSO) was from Merck. [^{35}S]Methionine (SJ.204) was from Amersham. These reagents are routinely used in this laboratory with good results.

B. Stock Solutions and Buffers for the First Dimension

These solutions are similar to those described previously by O'Farrell (1975).

Lysis buffer: 9.8 *M* urea
2% (w/v) NP-40
2% Ampholytes pH 7–9
100 m*M* DTT
keep at −80°C

Overlay buffer: 8 *M* urea
1% Ampholytes pH 7–9
5% (w/v) NP-40
5% β-mercaptoethanol
keep at −80°C

Equilibration buffer: 0.06 *M* Tris-HCl pH 6.8
2% SDS

5% β-mercaptoethanol or 100 m*M* DTT
10% glycerol
keep at room temperature

Acrylamide solution: 28.38% acrylamide
1.62% bisacrylamide
filter and keep at 4°C for not more than 2 weeks

NP-40 solution: 10% (w/v) NP-40 in H_2O
keep at room temperature

H_3PO_4 solution: 1 *M*, keep at room temperature

NaOH solution: 1 *M*, keep at 4°C, not more than 2 weeks

10× nuclease solution: 1 mg/ml DNase I
500 μg/ml RNase A
0.5 *M* Tris, pH 7
50 m*M* $MgCl_2$
keep at −80°C

Agarose solution: 1% agarose
0.002% bromphenol blue
in equilibration buffer
keep at 4°C

C. Stock Solutions and Buffers for the Second Dimension

Solution A (resolution gel): 30% (w/v) acrylamide
0.15% (w/v) bisacrylamide
filter and keep at 4°C for not more than 2 weeks

Solution B pH 8.8 (resolution buffer) for 1000 ml: 200 ml 1.5 *M* Tris-HCl
800 ml 1.5 *M* Tris-base
keep at 4°C

Solution C pH 6.8 (stacking buffer) for 1000 ml: 160 ml 1.5 *M* Tris-base
titrate to pH 6.8 with 6 N HCl
keep at 4°C

Solution D (stacking gel): 10% (w/v) acrylamide
0.5% (w/v) bisacrylamide
filter and keep at 4°C for not more than 2 weeks

5× running buffer (1000 ml): 30.3 g Tris-base
144 g glycine
make up to 0.1% in SDS when diluting
keep at room temperature

III. Experimental Procedures

A. *Labeling of Cells with [^{35}S]Methionine*

Cells can be grown in 0.25-ml flat-bottomed microtiter plates or in 35-mm dishes containing, respectively, 0.25 or 1.5 ml of Dulbecco's modified Eagle's medium (DMEM). To label cells grown in microtiter plates, the normal medium is replaced by 0.1 ml of homemade DMEM containing 1 mg/liter of cold methionine, 10% dialyzed fetal calf serum and 100 μCi of [^{35}S]methionine (Bravo and Celis, 1980a,b; Celis and Bravo, 1981; Bravo *et al.*, 1982). For labeling cells grown in 35-mm dishes, the medium is replaced by 1 ml of radioactive medium containing 500 μCi of [^{35}S]methionine.

If short term labeling is preferred (up to 3 h), DMEM without methionine is used because it increases significantly the radioactivity incorporated into proteins. This procedure is not recommended for long term labeling.

B. *Sample Preparation*

The two-dimensional gel system is sensitive to single charge changes that result in one protein giving several spots in the gel. Therefore, drastic conditions that cause chemical modifications of the proteins during sample preparation must be avoided.

When cells are grown and labeled in 0.25 ml microtiter wells the sample can be prepared with no special treatment. The labeling medium is aspirated carefully, and the cells are immediately lysed by adding 20–40 μl of lysis buffer. Samples are stored at −70°C as described by Bravo *et al.* (1982). Nucleic acids present in the sample in small amounts do not interfere with the first dimension.

If cells are grown and labeled in larger quantities, then treatment of

the sample with DNase and RNase is necessary. The following procedure (previously described by Garrels, 1979) should be done in the cold to avoid protein degradation. Cells scraped in 20 m*M* Tris, 2 m*M* $CaCl_2$, at pH 8.8, and dispersed through a narrow gauge needle are first treated with 0.3% final concentration of SDS to solubilize the proteins. Then nuclease solution containing DNase I and RNase A described in Section II,B is added, and the sample is left in the cold until the solution is no longer viscous. Following freeze-drying the sample is resuspended in lysis buffer and stored at −70°C. This procedure is also adequate for samples to be developed by silver staining (see chapters by Merril and Goldman and by Sammons).

C. Determination of Trichloroacetic Acid (TCA)-Precipitable Radioactivity in Samples

A reliable method for the determination of the radioactivity of the samples applied to the gels is needed for quantitative studies in order to describe the percentage of radioactive label in a given protein compared to the total number of counts applied to the gel.

An appropriate dilution of the radioactive sample containing 0.1 mg/ml of bovine serum albumin (BSA) is precipitated with 5 ml of cold 10% TCA, mixed vigorously, and left on ice for 30 min. Millipore filters (HA 0.45 μm) are previously soaked in 10% TCA containing 1% Casamino acids for about 10 min. The sample is then filtered and washed three to four times with cold 10% TCA. The filter is dried and counted in a scintillation counter. All assays are done in duplicate.

D. Procedures for the First Dimension

1. PREPARATION OF TUBES

It is important that the gel is firmly attached to the walls of the tube during electrophoresis; therefore the tubes must be carefully cleaned before being used. The glass tubes should be thoroughly washed in Decon, rinsed with water, then boiled in 0.1 *M* HCl for 30 min, rinsed with double-distilled water, and allowed to dry. Gel tubes should have an inside diameter of 1.2 to 3 mm and an appropriate length that may

vary from 12 to 20 cm. The tubes should all be of the same length and so should the gels, because these factors affect the reproducibility of the runs.

The bottom of the tubes is sealed with three layers of Parafilm to avoid leakage and to prevent the entry of air through the slightly porous Parafilm, which could affect polymerization. The tubes are marked to indicate the desired length of the gel (normally 1–2 cm from the top) and are placed vertically in a rack.

2. PREPARATION AND RUNNING OF THE FIRST DIMENSION

The procedure is essentially that described by O'Farrell (1975) and O'Farrell *et al.* (1977) with minor modifications (Bravo *et al.*, 1982).

To prepare 10 ml of gel mix for the first dimension add the following to a 125-ml side arm flask:

	IEF	NEPHGE
Urea (g)	5.5	5.5
Acrylamide solution (ml)	1.3	1.3
10% NP-40 (ml)	2.0	2.0
Distilled H_2O (ml)	1.7	2.0
Ampholyte pH 5–7 (ml)	0.6	—
Ampholyte pH 3–10 (ml)	0.12	—
Ampholyte pH 7–9 (ml)	—	0.25
Ampholyte pH 8–9.5 (ml)	—	0.25

The exact ampholyte composition can be varied according to the result desired.

Once all the reagents have been added, the flask is swirled until the urea is completely dissolved. This can be speeded up by placing the flask in a water bath at 37°C. The solution is degassed carefully by connecting the flask to a water pump. Then 7 μl of TEMED and 10 μl of 10% ammonium persulfate are added to the IEF solution. Twice as much TEMED and ammonium persulfate is necessary for the NEPHGE gels because the presence of basic ampholytes affects polymerization. The solution is then immediately loaded into the tubes using a long, narrow gauge blunt end needle. To avoid trapping air bubbles, the tip of the needle is inserted to the bottom of the tube and withdrawn slowly as the acrylamide mixture runs in. The tubes are filled up to a specified mark so that all gels are of the same length. The gel mixture is overlayed with 10 μl of H_2O and allowed to polymerize for at least 2 h. The Parafilm is then