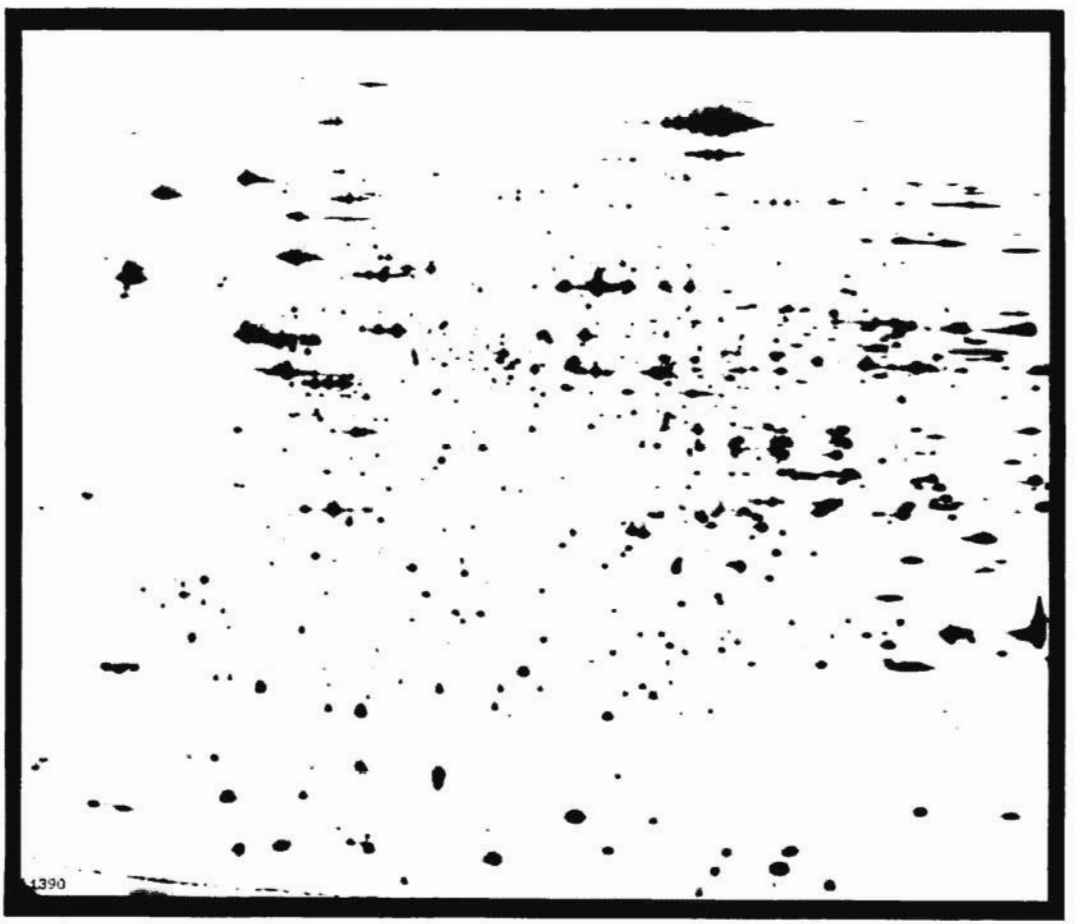
Two-Dimensional Electrophoresis

Operation of the ISO-DALT® System

by

LEIGH ANDERSON



Second Edition

1991

Large Scale Biology Press

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1 Preface

The ISO-DALT® system for two-dimensional (2-D) electrophoresis was developed by Norman and Leigh Anderson and their coworkers, first at the Argonne National Laboratory and later at Large Scale Biology Corporation (LSB). This effort began in 1977 as an attempt to increase the reproducibility and decrease the labor-intensiveness of O'Farrell's original technique (**1**) for 2-D electrophoresis of proteins. Since the appearance of the first two publications describing the ISO-DALT system in 1978 (**2,3**), the technique has been widely adopted and continues to be refined through automation, engineering improvements and the development of standards (**4,5,6**). Current versions of the ISO-DALT system components are available through Hoefer Scientific Instruments, San Francisco. Angelique™ and other more automated components are produced by LSB directly.

As a supplement to the published literature, a series of informal manuals was prepared describing recipes and detailed laboratory procedures that facilitate use of the system for standardized 2-D work. The current ISO-DALT manual is the ninth edition of this material and represents the state of art as of September 1991. Versions I-IV were untitled, prepared for our own use and for use by those who came to Argonne to learn the technique. In 1979 an enlarged Version V became the first of three such manuals to be published as an Argonne National Laboratory Report (**7,8,9**), allowing wider distribution and appropriate citation. Following a sixth edition in 1981, new sections

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1) High-Resolution Two-Dimensional Electrophoresis of Proteins. P. H. O'Farrell, J. Biol. Chem. 250: 4007-4021, 1975.

2) Analytical techniques for cell fractions. XXI. Two-dimensional analysis of serum and tissue protein: Multiple isoelectric focusing. Anderson, N.G. and Anderson, N.L., Anal. Biochem. 85: 331-340, 1978.

3) Analytical techniques for cell fractions. XXII. Two-dimensional analysis of serum and tissue proteins: Multiple gradient-slab electrophoresis. Anderson, N.L. and Anderson, N.G., Anal. Biochem. 85: 341-354, 1978.

4) Analytical techniques for cell fractions. XXIV. Isoelectric point standards for two-dimensional electrophoresis. Anderson, N.L. and Hickman, B.J., Anal. Biochem. 93: 312-320, 1979.

5) Analytical techniques for cell fractions. XXVII. Use of heart proteins as reference standards in two-dimensional electrophoresis. Giometti, C.S., Anderson, N.G., Tollaksen, S.L., Edwards, J.J., and Anderson, N.L., Anal. Biochem. 102: 47-58, 1980.

6) The use of carbamylated charge standards for testing batches of ampholyte used in two-dimensional electrophoresis. Tollaksen, S.L., Edwards, J.J., and Anderson, N.G., Electrophoresis 2: 155-160, 1981.

7) Argonne National Laboratory Report ANL-BIM-79-2.

8) Argonne National Laboratory Report ANL-BIM-81-1.

9) Argonne National Laboratory Report ANL-BIM-84-1.

on silver staining, Western transfers, ACIDO's and BASO's were added and this seventh edition appeared in May 1984. Version VII in the Argonne series was written by Sandra Tollaksen, myself and Norman Anderson, and provided the scaffold for the construction of the revised manual first published by LSB(**10**).

The LSB-published ISO-DALT manual (the eighth in the series) attempted to retain the practical flavor of the original series, while completely rewriting the text to improve organization, clarity, and rigor. A number of new sections were included. Recipes were reworked to cover both the original 7"x7" slab format and the more recently introduced 8"x10" size. Gravimetric recipes for the stock solutions were offered as a superior method of achieving quality control. Improved gel casting methods were covered in some detail and a brief discussion of computer analysis of 2-D gels was included. Sharron Nance and Jean-Paul Hofmann contributed technical updates to several sections. Finally, we added substantial new material on the justification for particular approaches and emphasized those aspects requiring special care if results are to be compared between labs. While much of this material was of general utility, the specific instructions contained were intended for use only with the ISO-DALT systems manufactured by LSB, holder of the ISO-DALT trademark, for sale exclusively by Hoefer Scientific Instruments.

The present (ninth) edition has been updated in several respects. Operation of the new Hoefer ID125 version of the Iso apparatus is covered in addition to the original design, and several improvements in slab gel technique are covered as well: the Angelique computer-controlled gradient maker, Hoefer's ID350 pump-assisted gradient maker, the Equalizer™ slab overlay device and Wedgies™ gel interface chocks (a replacement for melted agarose).

A partial list of those who have contributed to the development of the ISO-DALT system over the years includes the following people, all of whom are gratefully acknowledged. None are accountable for any errors, oversights or opinions in this document, since these originate with the author.

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10) Some portions of this manual are reproduced in modified form from Argonne National Laboratory Reports ANL-BIM-72-2, ANL-BIM-81-1 and ANL-BIM-84-1 with permission of the Laboratory and the authors.

Sandra Tollaksen	Sharron Nance	Norman G. Anderson
Anne Gemmell	Carol S Giometti	Sharon Pascoe
Barbara Hickman	Karen Willard-Gallo	Bill Cole
Russell Tracy	Fred Giere	Tom Doody
Nakwon Cho	Harold Harrison	Bill Eisler
Leo Kirkel	Jesse Edwards	Don LeBuis
John Taylor	Michel Zivy	Terry Pearson
Peter Spragg	Dave Bateman	Ivan Lefkovitz
Beth Anderson	Walter Schick	Tom Jones
Jean-Paul Hofmann	Sean Gallagher	Ricardo Esquer-Blasco

Special thanks to Peter Hoefer, a man of so many good ideas and such a commitment to providing quality. Thanks also to Sharron Nance, Jean-Paul Hofmann, John Taylor, Constance Seniff and Angie Lackey for reading draft versions, and especially to Ricardo Esquer for contributing several sections on current practice to this edition.

This manual was produced using Lotus Manuscript®, the Apple LaserWriter®, and Microsoft Paintbrush®.

In the sections that follow, paragraphs that involve an action on the part of the user are generally marked with a bullet (•). The Iso, Dalt and Angelique chapters each conclude with a section of condensed instructions for routine use.

> The technology of two-dimensional electrophoresis involves use of both high voltage electricity and acrylamide, a substance known to be a neurotoxin. Each user must be aware of these potential hazards and take proper precautions to avoid injury.

2 Overview

Two-dimensional electrophoresis is the only method available for routinely separating, detecting and quantitating hundreds or thousands of proteins. Because of its tremendous resolving power, it offers the possibility to probe very complex patterns of gene expression, to investigate and interpret subtle alterations in protein structure and to search for genetic variation at large numbers of loci simultaneously. Two-dimensional electrophoretic protein mapping is unique in that it encourages the user to make discoveries in the larger sphere of "unknown" proteins, rather than limiting the approach to known entities.

Roughly 5,000 scientific papers have been published using 2-D gels since 1975. Substantial contributions have been made in many areas. However, progress in using and sharing access to the vast amount of information that can be generated by 2-D mapping has been impeded by two main limitations. The first is an insufficiency of good computer analysis systems, a problem which is now being rapidly overcome through the use of LSB's Kepler® and other recently-introduced gel analysis workstations. The second, and in some ways more fundamental, limitation has been the lack of gel standardization between laboratories. Although the effort involved in changing 2-D systems can be significant, the benefits derived from the ability to exchange data are tremendous.

Beginning with O'Farrell's paper in 1975, many people set up the 2-D system, generally adapting it to locally-available equipment and reagents (such as ampholytes and sodium dodecyl sulfate [SDS]), and adding small improvements aimed at convenience. Unfortunately, the O'Farrell technique is critically dependent upon exactly these small details: upon equipment geometry, reagents and procedure. Thus, while it is generally possible to get a "good" 2-D gel starting with any permutation of these factors, the specific pattern of molecules obtained is not likely to be directly comparable to that produced in another lab that began its own technical evolution independently.

Three elements are essential to doing good 2-D gel work: a reproducible approach to running the gels; a capability to run many gels when needed; and a level of convenience that prevents "burn-out" of the people who do the gel work. The need for reproducibility is now obvious, since investigators in different labs have increasing amounts of interesting information to exchange on hitherto "unknown" proteins once they can establish that they are referring to the same molecule. Many gels are needed to allow screening of more

experimental variables, to provide for reasonable statistics (in both quantitative and qualitative experiments), and to maximize the batch size within which reproducibility is likely to be most rigorously preserved. Convenience becomes a factor as gel runs increase.

Happily, these three factors work together to produce good gels. In practice, a system that can run many gels conveniently is often the most reproducible. This is a well-known feature of mass production processes: the best quality often comes from the most efficient (and cheapest) process. In the 2-D gel world, running more gels more often usually means better gels.

The ISO-DALT® system was initially designed to process batches of 10 gels at a time. This was later raised to 20 gels, which is now the predominant batch size in use. Twenty gels turns out to be a convenient number for a user to process at each step. Perhaps more important, 20 gels provides greatly expanded scope in experimental design. Very few useful experiments involve only one control and one or two experimental samples; more often a set of ten samples run in duplicate is appropriate (**11**).

Users should therefore take comfort in the fact that it is often less effort to run 20 gels with the ISO-DALT system than it is to set up and run 2 or 4 using equipment designed for 1-D work.

A consensus has gradually emerged in favor of a standard way of presenting 2-D gel data for publication (**12**). Almost all investigators now show gels with acid isoelectric points (pI's) to the left and high sodium dodecyl sulfate molecular weights at the top. This results in a system of pI and molecular weight values that run according to the Cartesian convention and facilitates the use of the charge and molecular weight standards described here. This convention is highly recommended for all users.

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11) Consider for example a comparison of lymphocytes from control and leukemic patients. If 500 proteins are observed, about five changes will be observed at a significance level of $P<.01$ by chance alone. Such a significance level is usually considered appropriate in biological experiments that examine one or a few variables. Two-D gels clearly present an exceptional situation in that hundreds of independent variables are involved. Replication of gels for each sample, or replication of similar samples, is of tremendous importance in obtaining valid results at this level of complexity.

12) Introduction. Some perspectives on two-dimensional protein mapping. Anderson, N.L., and Anderson, N.G., Clin. Chem. 30: 1898-1905, 1984.

2.1 Recipes for Gels and Solutions

Since the recipes used for gel work are generally made up many times without significant change, it is worth some trouble to compute recipes that are easy to prepare reproducibly. It turns out that most of the solution recipes include both solid and liquid components; these have conventionally been made up by weighing the solids and measuring the liquids (and the final solution) volumetrically. In this manual, we include, in addition to the standard format recipes, some versions set up for making the solutions by weight. This requires knowledge of the densities of various components and of the final solution; these densities have been taken into account in developing the recipes here. We believe the weight-weight method is to be preferred when a good digital balance is available, since it is easier to reproduce a given accuracy in weighing than by volumetric measurement. One also ends up with fewer vessels to keep clean.

2.2 Useful Sources of Additional Technical Information on 2-D Gels

In addition to more than 5,000 published papers using 2-D electrophoresis of proteins, there is a variety of books and chapters on 2-D electrophoresis technique, most of which contain some unique and useful information. We list below some useful sources. Those that describe the ISO-DALT system specifically are marked with a dagger (†) and those available directly from Hoefer Scientific Instruments with a double dagger (‡).

Use of large-scale two-dimensional ISO-DALT gel electrophoresis system in immunology. †

Lefkovits, Ivan, Young, Patricia, Kuhn, Lotte, Kettman, Jack, Gemmell, Anne, Tollacksen, Sandra, Anderson, Leigh, and Anderson, Norman

In: Immunological Methods, Vol.III, Lefkovits, Ivan and Pernis, Benvenuto, eds., Academic Press, Inc., Orlando, Chapter 11, pp 163-185, 1985. (ISBN 0-12-442703-0)

Use of high-resolution two-dimensional gel electrophoresis for analysis of monoclonal antibodies and their specific antigens. †

Anderson, N.L. and Pearson, T.W.

Methods in Enzymol. 92: 196-200, 1983.

Applications of two-dimensional gel electrophoresis in the clinical laboratory. †

Tracy, Russell P. and Anderson, N. Leigh

In: Clinical Laboratory Annual, Homburger, Henry A. and Batsakis, John G., eds., Appleton-Century-Crofts, Connecticut, pp 101-130, 1983. (ISBN 0-8385-1142-2)

Operation of the ISO-DALT system. Seventh edition. †

Tollaksen, Sandra L., Anderson, N. Leigh and Anderson, Norman G.

Argonne National Laboratory Report ANL-BIM-84-1, May 1984.

Two-Dimensional Electrophoresis and Immunological Techniques. †

Bonnie S. Dunbar

Plenum, 1987.

Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications

Julio Celis and Roderigo Bravo, eds.

Academic Press, New York, 1984. (ISBN 0-12-164-720-X)

Electrophoresis: Theory, Techniques, and Biomedical and Clinical Applications

A.T. Andrews

Oxford University Press, 1981. (ISBN 0-19-854626-2)

Gel Electrophoresis of Proteins: a practical approach ‡

B. D. Hames and D. Rickwood, eds.

IRL Press Limited, London and Washington, D.C., 1981. (ISBN 0-904147-22-3)

Isoelectric Focusing: Theory, Methodology and Applications

P. G. Righetti

Series: Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 11, T.S. Work and R.H. Burdon, series eds., Elsevier Biomedical Press, 1983. (ISBN 0-444-80467-6)

2.3 Collections of Papers Describing 2-D Applications

Special Issue: Two-Dimensional Electrophoresis (1982) ‡

A collection of 51 papers on 2-D electrophoresis presented at an international meeting on "Clinical Applications and Developments in Two-Dimensional Electrophoresis" and published as a volume of the journal Clinical Chemistry.

Clin. Chem. Volume 28, No. 4, pp.737-1092 (1982).

Special Issue: Two-Dimensional Electrophoresis and Protein Mapping (1984) ‡

A collection of 36 papers on 2-D electrophoresis presented at the "Third International Symposium on Two-Dimensional Electrophoretic Protein Mapping" and published as a volume of Clinical Chemistry.

Clin. Chem. Volume 30, No. 12, pp.1897-2108 (1984).

Electrophoresis '79

Proceedings of the Second International Conference on Electrophoresis, Munich. Contains 13 papers on 2-D electrophoresis.

B. J. Radola, ed., Walter de Gruyter, Berlin, New York, 1980. (ISBN 3-11-008154-7)

Electrophoresis '83

Proceedings of the Fourth International Conference on Electrophoresis, Tokyo. Contains 21 papers on 2-D electrophoresis.

H. Hirai, ed., Walter de Gruyter, Berlin & New York, 1984. (ISBN 3-11-009788-5)

Electrophoresis '86

Proceedings of the Fifth Meeting of the International Electrophoresis Society, London. Contains 37 papers on 2-D electrophoresis.

M. J. Dunn, ed., VCH, Weinheim, W. Germany & Deerfield Beach, Florida. (ISBN 0-89573-583-0)

Progrès Récents en Electrophorèse Bidimensionelle (Recent Progress in Two-Dimensional Electrophoresis)

Proceedings of a symposium on 2-D electrophoresis held in 1985 at Pont-à-Mousson (near Nancy) France. Contains 52 papers on 2-D electrophoresis.

Galteau, M.-M. and Siest, G., eds., Presses Universitaire de Nancy, France, 1986. (ISBN 2-86480-248-1)

Two-Dimensional Electrophoresis

Proceedings of an international 2-D electrophoresis meeting held in Vienna, Austria.

Endler, A.T., and Hanash, S., eds., VCH Verlagsgesellschaft mbH, Weinheim, 1989.

2-D PAGE '91

Proceedings of the International Meeting on Two-Dimensional Electrophoresis held in London, England, July 16-18, 1991.

Dunn, Michael J., ed., Department of Cardiothoracic Surgery, National Heart and Lung Institute, London, 1991. (ISBN 0-9517684-0-9)

2.4 The Initial Papers

Two-dimensional electrophoresis of proteins in the form we know it today developed principally from techniques presented by Pat O'Farrell in 1975. Pat was a graduate student at Boulder, Colorado at the time, and the story of the difficulty he experienced in getting his paper accepted is a legend. At almost the same time, three other labs were also describing high-resolution 2-D systems (Klose, Scheele, and Iborra and Buhler). Thus the following four papers constitute, in a sense, the origin of the modern era in 2-D technique. They are all worth reading today, especially O'Farrell's.

High Resolution Two-Dimensional Electrophoresis of Proteins

O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007-4021.

Protein Mapping by Combined Isoelectric Focusing and Electrophoresis in Mouse Tissues. A Novel Approach to Testing for Induced Point Mutations in Mammals.

Klose, J. (1975) Humangenetik 26, 231-243.

Two-dimensional Gel Analysis of Soluble Proteins. Characterization of Guinea Pig Exocrine Pancreatic Proteins.

Schele, G.A. (1975) J. Biol. Chem. 250, 5375-5385.

Protein Subunit Mapping. A Sensitive High Resolution Method.

Iborra, G and Buhler, J.-M. (1976) Analyt. Biochem. 74, 503-511.

3 Record Keeping

3.1 What Needs to Be Recorded

Since there are many details of experimental procedure that can influence the quality and reproducibility of the 2-D patterns, it is critical to minimize any deviation from the standard protocol and record any deviations that do occur. This document contains a series of forms designed to assist in successful execution of the 2-D procedure and to prompt the user to record all foreseeable problems. Hence the forms contained here are meant to be filled out completely during the course of each run.

3.2 Preservation of Records

As part of our approach to GLP procedures, it is necessary that no important records are lost. All standard forms must make their way back to the appropriate laboratory notebooks, and these notebooks must be copied on a regular basis to provide archival copies in case of loss of the originals.

4 Sample Preparation

The objectives of sample preparation procedures used in advance of 2-D electrophoresis are to solubilize as many proteins as possible, to prevent any subsequent chemical modification of the proteins (particularly proteolysis), and to eliminate or counteract any substances that might interfere with the 2-D separation (such as DNA or plant phenols).

Several interesting observations were made in the very early days of 2-D work that deserve recapitulation. First, although SDS is an extremely powerful protein denaturing agent, it turns out that a combination of a weak nonionic detergent (Nonidet P-40; NP-40) and a high concentration of urea (9M) is even stronger. Thus O'Farrell's original solubilizing solution is hard to beat as a means of stopping all enzymatic activity in a sample and of "unraveling" the proteins. The single modification that has proved most useful involves using a higher pH in order to take the sample far outside the pH optimum of cellular proteolytic enzymes (see "pink mix" in recipe section).

Second, the isoelectric focusing first dimension separation is adversely affected by the DNA present in most cellular samples. Once again, O'Farrell found an excellent method of removing cellular DNA by means of complexation with positively charged ampholytes. Wide range ampholytes added to the sample solubilizer replace the histones and other proteins that keep DNA condensed in living cells, thereby preventing an "explosion" of the DNA to form a viscous and restrictive gel. In the modified high-pH solubilizer recipe used here, it is necessary to use alkaline range ampholytes to achieve this effect.

Lastly, it has proven possible to analyze unfractionated samples from a wide range of sources, thus generally eliminating the necessity to prepare subfractions of cells or tissues. Fractionation procedures carried out before denaturation always involve some risk of protein modification, particularly when there is the possibility that granulocytes or other cells with major degradative functions are present. Since the resolution of 2-D gels is generally sufficient to separate the starting sample, sample fractionation is now considered advisable only when it serves some other purpose, such as identifying mitochondrial or cytoskeletal proteins, or enriching some specific low-abundance subset.

4.1 Solubilization

A sample to be prepared for two-dimensional electrophoresis using the ISO-DALT® system is first mixed with a solubilizing solution (a "mix") before separation in the first dimension by isoelectric point. Most work is possible using the methods of sample preparation described in this section. There are instances (such as bone), however, that require specialized techniques outside the scope of this manual.

The mixes used here are made up in bulk (100-1000 ml) and stored frozen at -70°C in microfuge tubes as 1 ml aliquots. A fresh tube is thawed for each experiment.

4.1.1 Tissue Samples and Most Cultured Cells: Standard Urea Mix

The standard high-pH urea/NP-40 mix (see recipe section) works well with solid tissue samples and cultured cells such as fibroblasts and lymphoid cells. This solubilizer is colloquially known as "pink" mix because it was stored as aliquots in pink microfuge tubes (other mixes were stored in other color-coded tubes).

For suspension cells the following procedure was developed. Pellet the cells in a capillary-bore microfuge tube (Walter Sarstedt Co.). The pellet should be no more than 1 mm deep in the capillary section. Remove the liquid medium from above the cells completely with a needle aspirator. Then, using a 100 µl Hamilton syringe containing the urea solubilizer, gently (but quickly) "jet" resuspend the cells in solubilizer. By doing this evenly, each cell is exposed to full strength solubilizer the instant it is "uncovered" in the pellet. If the erosion of the pellet is uneven, a glob of cells will remain in which solubilizer has gradually penetrated a mass, leaving a DNA gel. After successful solubilization, spin out the nuclear remnants in a microfuge (30 sec). You will get an insoluble pellet much smaller than the original cellular pellet.

Cultured cells that grow attached to the bottom of a well or flask can be solubilized (after removal of medium and rinsing with phosphate-buffered saline to remove serum, etc.) by flowing a small volume of solubilizer over the inclined bottom surface. The solubilizer can be flowed down repeatedly to make sure that all attached cells are removed. The procedure can be done conveniently using either a Hamilton syringe or an Eppendorf-type pipette (if care is taken to avoid foaming during pipetting).

For tissue portions, use from 3-10 µl of solubilizer per mg of tissue. In general, the sample should be homogenized in solubilizer as rapidly as possible using a classical pestle homogenizer. In critical applications, the sample can be frozen and ground in a dry-ice-cooled steel mortar and pestle, with solubilizer added to the frozen powder after transfer to a plastic vessel. Alternatively, the tissue can be sectioned using a frozen microtome and thin sections solubilized in the wells of a microtiter plate.

From 10 to 30 µl of the urea mix may also be mixed with 10 µl of a concentrated liquid protein sample.

Do not heat the urea mix. Heating will result in creation of cyanates (leading to carbamylation of proteins and ampholytes), as well as other reactive compounds. As a general rule, it is important to solubilize as quickly as possible to prevent DNA gel formation.

4.1.2 Plasma Proteins

Sodium dodecyl sulfate mix (see recipe section) is quite useful for serum, plasma, cerebrospinal fluid (CSF), or amniotic fluid: the small amount of SDS aids in transporting proteins into the gel. Ten µl sample is mixed with about 20-30 µl SDS mix (making a 1:3 or 1:4 dilution). The sample plus mix may be heated on a 95° C heating block for 5 min to solubilize it. [Be careful, however: this heating step is not generally necessary and can in some cases cause cleavage of particular proteins.] After focusing, the SDS ends up as a bulb at the acid end of the Iso gel, provided that the gel is not overloaded with SDS (about 10 µl of the sample as described can be accommodated).

4.1.3 Plant Proteins

Plant samples represent one of the most challenging extraction problems (**13,14**). They often contain phenols and other organic compounds in cellular vacuoles that are broken in the course of protein extraction. Many

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13) Study on nuclear and cytoplasmic genome expression in wheat by two-dimensional gel electrophoresis. 2. Genetic differences between two lines and two groups of cytoplasms at five developmental stages or organs. Zivy, M., Thiellement, H., de Vienne, D., and Hofmann, J.-P., Theor. Appl. Genet. 68: 335-345, 1984.

14) Two-dimensional gel electrophoresis of proteins for genetic studies in Douglas fir (pseudotsuga menziesii). Bahrman, Nasser, de Vienne, Dominique, Thiellement, Hervé, and Hofmann, Jean-Paul, Biochemical Genetics 23: 247-255, 1985.

of these compounds bind to or otherwise modify proteins, necessitating special methods for successful sample preparation. The method presented here (developed by LSB staff) has been used extensively for extraction of protein samples from leaves and shoots.

Frozen plant tissue samples are ground to a fine powder using a mortar and pestle sitting in liquid nitrogen. The powder is scraped into a pre-weighed 50 ml centrifuge tube and lyophilized for approximately 22 hours. After lyophilization, the sample is weighed (in the tube) and dry weight determined. Next, each sample is taken up in 15 ml of a 10% trichloroacetic acid (TCA) solution in acetone (with 0.07% mercaptoethanol), shaken and placed in a -20°C freezer for 30 minutes (shaken once during this period). The sample is then centrifuged for 15 minutes at 3000 rpm in a refrigerated centrifuge (e.g., Beckman J6). The supernatant is poured off and the pellet resuspended in 50 ml of acetone with 0.07% mercaptoethanol. This second extraction is also carried out in the -20°C freezer, this time for 20 minutes with agitation at the midway point. The samples are again centrifuged as above and the supernatant discarded. Tubes are placed upside down on blotting paper for a few minutes to drain, then placed upright and the pellets allowed to air dry for 15-20 minutes. UKS extraction mixture (see recipe section) is added to the pellet (40 µl/mg dry weight) and mixed with a glass rod for 2 minutes. The sample is then placed in a microfuge tube and spun for 5 minutes in a microfuge. The supernatant is collected and placed in a clean microfuge tube for storage at -70°C until needed for a 2-D run.

4.2 Post-Solubilization Cleanup

Many sample types contain DNA and insoluble cell debris. In order to prevent "clogging" of the surface of the Iso gel, it is useful to centrifuge the sample, saving the supernatant for analysis and discarding the pellet. In most cases, a microfuge spin of 30 sec suffices to pellet the nuclear DNA. Using a capillary-bottom microfuge tube will usually prevent the pellet from becoming resuspended inadvertently, and therefore make it possible to freeze and store the sample in the same tube in which it is spun. A more rigorous procedure, particularly useful for samples made from solid tissues, is to spin the sample for 1 hour at 100,000 g in a typical ultracentrifuge (or 15 min at 400,000 g in a machine like the Beckman TL-100).

4.3 Sample Storage

Samples solubilized using the procedures described above should be stored frozen at -70°C or lower, in which case they are stable indefinitely. The freezing should be relatively rapid in order to avoid the formation of urea crystals and associated depletion of urea in the liquid phase. Samples should not be kept refrigerated, or at -20°C, for any length of time; they are, however, stable at room temperature for hours. Most samples can be repeatedly thawed and re-frozen.

5 Iso: the First Dimension

The first dimension separation of the classical O'Farrell 2-D procedure involves isoelectric focusing (IEF) under denaturing conditions in small diameter tube gels. IEF separates proteins according to their isoelectric points by causing them to migrate in an electric field through a pH gradient: when each protein reaches a point in the gradient whose pH is equal to the protein's characteristic isoelectric point, the protein has no net electric charge and hence stops moving (it "focuses"). Since isoelectric point is determined mainly by amino acid composition (and to some extent by sequence), IEF separates proteins principally by chemical (as opposed to size) characteristics. The pH gradient required is generated dynamically by electric field-induced movements of heterogeneous mixtures of organic compounds known as "ampholytes". An alternative method capable of establishing permanent, fixed pH gradients using immobilized charged groups (**15**) is being explored but has not yet achieved widespread use in 2-D systems.

Focusing is used because of its high resolving power; denaturing conditions are used because protein subunits resolve better than assembled complexes; and tube gels are used because they afford high symmetry and thus avoid the problems that lead to waves in conventional flat bed IEF gels. The Iso system was designed to run tube gels whose diameter is the same as the thickness of the slab gels to be used for the second dimension, thus eliminating the need for beveled plates: the Iso gel slips between the slab gel plates to lie directly on top of the slab gel.

5.1 Ampholytes: Think Hard About Standardization

Most commercially-available ampholytes are usable for 2-D electrophoresis; however, none are yet routinely quality-controlled by means of 2-D. Since 2-D methods present a much more stringent reproducibility requirement than 1-D IEF, you will find variation among batches of a given commercial ampholyte product (e.g., LKB 3-10) and large differences between similar products offered by different producers (e.g., LKB, Pharmacia, Serva, BDH and BioRad). Chemically, this is a difficult problem and one not likely to be quickly solved.

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15) Immobilines® produced by LKB.

It is therefore very important to consider how to standardize ampholytes before beginning any large-scale effort, particularly one that may continue over an extended period of time. Ideally, one should also make arrangements to standardize across laboratories when data is to be exchanged.

At present, there is really only one way to achieve the standardization required: carefully choose a good ampholyte batch or mixture and acquire a lot of it. If a long-term project will require 5,000 gels and last two or three years, then about 190 ml of ampholyte will be required and 500 ml would provide a possibility for future extension of the work. This sounds expensive (and it can be) but the several thousands of dollars it may cost will be spent anyway, and such a one-time outlay represents an economy compared to the labor costs involved in having to make a batch change in the middle of a study. Ampholytes are reported to be stable indefinitely when kept cold (they can be frozen without harm) and sterile (contamination of ampholytes can cause growth of microorganisms and consequent degradation). Make arrangements either to buy the required amount as one lot (substantial savings can be had if you will take the material from the manufacturer in one large bottle and aliquot it yourself using sterile technique), or otherwise ensure that someone else (a multi-lab project coordinator or a departmental gel syndicate) has a sufficiently large, homogeneous lot available and will provide what you need. In any case, be certain that you are working with a known, homogeneous lot of ampholyte: ampholytes represent the largest single source of gel-to-gel variation in the 2-D procedure and a change in mid-stream can cost weeks of testing and frustration. Plan for the future.

In collaboration with Hoefer and BDH, LSB has tested a number of batches of the BDH 4-8 range ampholyte for 2-D use. This material appears to be an excellent choice for most mammalian tissues, since the pH gradient it produces is almost uniformly covered with cellular proteins. Use of a nominally wider gradient (e.g., 3.5-10) yields little extra information in a 2-D application, since there are very few proteins more acidic than the 4-8's acid limit, and few more basic proteins are resolvable due to cathodic drift.

5.2 Apparatus

The Iso apparatus (see Figures in section 5.18) is designed to allow parallel isoelectric focusing of 20 samples in small bore (generally 1.5 mm)

glass tubes. Its major advantages are the ease of casting the gels using hydrostatic displacement of gel solution; the ability to run 10" long gels (or any shorter length); the use of removable tubes (facilitating cleaning and tube replacement); an evenness of temperature due to the water-jacketing effect of the lower buffer chamber; and the compactness of the device.

5.2.1 Tubes

The tubes used are small bore (1.5 or 1.0 mm inside diameter [ID]), thick wall (4 mm OD) borosilicate glass tubes. Two lengths are used most commonly: 8" and 11", suitable for 7" and 10" gel rods, respectively.

Depending upon the method chosen for cleaning the system, the tubes can either be demounted after every run or left in the apparatus. If the tubes are cleaned in the apparatus, care should be taken to ensure that all the inside surface of the tube is cleaned, including the top region where the sample is applied.

In the past it was conventional to clean the tubes in hot chromic acid: the tubes are removed from the Iso and placed in a glass or teflon vessel (e.g., a graduated cylinder) filled with chromic acid that has been heated in a microwave oven (or other convenient warming system). Chromic acid is not compatible with lucite plastic and thus the tubes should not be chromic acid cleaned while they are in the apparatus.

More recently it has become customary to use advanced detergent mixtures like RBS-35 concentrate (Pierce Chemical Co.). It should be emphasized that, for best results, tubes should still be removed and cleaned after each run. When using detergent, however, it is possible, although not preferred, to clean the Iso apparatus in one piece by filling the entire Iso (both upper and lower buffer chambers) with cleaning solution. Then, by lifting the upper chamber occasionally and allowing it to sink again, flow can be generated through the tubes, creating a cleaning action.

When deciding which method of cleaning to use, the advantages of the detergent cleaning procedure over the more dangerous use of chromic acid should be taken into account. Use of detergent is recommended here.

Regardless which method is chosen, the tubes should be extensively rinsed with very pure water to remove all traces of cleaning agent and then dried thoroughly prior to gel casting. Drying requires passage of considerable air through the tubes, not just sitting in a dry environment for a day. The best method is to draw room air through the tube by holding a flexible

tube connected to vacuum against one end. Use of house compressed air is generally not good because of the presence of oil and dirt in the high pressure air.

Tubes fit into the upper chamber through silicone rubber grommets, which are in turn held in place by a grommet retaining plate (not removed between runs).

5.2.2 The New ID 125 Tube Gel Unit

In this new model, the upper buffer chamber no longer has support legs. Instead the upper chamber attaches to the lower chamber, secured by hooks built into the upper chamber. The upper chamber can hang either inside or outside the unit. Place it on the outside for loading the monomer into the casting boat and on the inside for casting the tube gels and for isoelectric focusing (see following sections). As a result of this new design the parameters of the apparatus have been slightly changed:

- The ID 125 upper buffer chamber is smaller than the one in the old model (it holds 200 ml only).

- The lower electrode is mounted on the exterior side of the upper chamber on a projecting ridge. To have the level of the lower buffer above its electrode, fill with about 3,260 ml of water (noting the correct fill lines).

Specific directions for this ID 125 model are given in the following sections.

5.2.3 Electrodes

Both platinum electrodes are mounted on the inner assembly comprising the upper buffer chamber, gel tubes and support legs (Figure 5.18.1). The upper electrode (typically the cathode in IEF) is mounted on the floor of the the upper chamber, between the two rows of gel tubes.

The lower electrode (usually connected as the anode in IEF) is mounted at the top of a bar connecting the two legs below the upper chamber (or projecting down from the upper chamber in the ID125). It is important that the buffer level in the lower (outer) chamber is above the level of this electrode in order that electrical contact is established between the tube ends (across the gel). Thus the lower buffer level should be between the lower electrode and the bottom surface of the upper chamber (about 3 liters of lower electrode buffer in the LSB version; 3.26 liters in ID

125). Do not let the lower buffer level touch the bottom of the upper chamber, as this can encourage electrical leakage around the sealing grommets. This level also ensures that the gel tubes are completely immersed in lower buffer and hence run at uniform temperature.

5.2.4 Power Supply

A regulated DC power supply capable of delivering at least 800V at 10mA is required to run 7" Iso gels, and a 1500V supply is preferable if 10" Iso's are to be run. The Hoefer Scientific Instruments PS 1500, or equivalent, will do the job nicely.

5.2.5 Additional Useful Gadgets

The following small pieces of apparatus are most useful in running the Iso system:

- A 10 µl and a 100 µl Hamilton (or equivalent) syringes for sample loading and to remove the air on the top of the tubes before prefocusing respectively.

- A 1 ml plastic (tuberculin) syringe with tapered fitting (needle removed), to which is fitted an Eppendorf-type yellow pipette tip cut off at the top so as to mate with the syringe taper. This device is used for removing the gels from the tubes after the run.

- A plastic 500 ml graduated cylinder in which the tubes can be washed. Plastic is preferred so as to prevent chipping of the tube ends when they are dropped into the cylinder with cleaning solution.

5.3 Assembly for Gel Casting

Clean glass tubes are mounted in the upper chamber before gel casting and remain in place throughout subsequent steps until the gels are finally unloaded. The tube gels are cast in one step using a simple displacement technique. The method (hydrostatic displacement) involves forcing the gel solution in the boat up into the glass tubes by the pressure of overlying water: as the assembly is lowered into the outer box, gel solution rises in the tubes. (Figures 5.18.2-5.18.4).

• Mount the glass tubes in the floor of the upper buffer chamber by gently pushing them up through the silicone rubber grommets. A small amount of glycerol is useful to lubricate the grommets during this process: apply a little to the bottom of each grommet using a "Q-tip" dipped in glycerol. Tubes should then slide in easily. Be careful when inserting these tubes: too much pressure can break the tube and could injure a hand.

• A short acrylic rod with a hole in one end is provided as a gauge to set all tubes to the same height in the apparatus. Start with all the tubes protruding about .5" into the upper chamber (i.e., further than they will for the run). Press the tubes down, one by one, with the upper tube end in the hole of the rod. The result will be that all the tubes will extend into the upper chamber to the same height.

• Fit the gel casting boat around the two rows of glass tubes while the screws holding the boat together are loose. One row of tubes goes in each of the two slot-like chambers of the gel casting boat (Figure 5.18.2).

• Tighten the boat assembly screws to both seal the boat and grasp the tubes. Be careful that none of the tube ends is jammed against the gasket forming the bottom of the boat: this could prevent gel rising in that tube. The tube ends should be about 1 mm above the gasket. Once the boat is assembled and the screws tightened, the boat is supported by the tubes themselves. This approach permits the added flexibility of using shorter tubes, if desired, without necessitating any other change in the equipment.

• In Hoefer's ID 125 model, "c"-section clamps replace the screws used to attach the casting boat to the bottom of the glass gel tubes. Place the upper buffer chamber upside down so the glass gel tubes extend upward. Spread the two rows of gel tubes apart and slide them into the appropiate grooves cut in the center section of the acrylic casting boat. With the grooves along each side facing out, place a face plate onto the seated tubes. While firmly holding the face plates in place, slide the plastic "c"-section clamps onto first one edge and then the other. A total of four clamps are required, two for each face plate.

5.4 Preparation of Gels: Conventional O'Farrell Gels

• Fill the empty lower (outer) chamber of the Iso system with approximately 3.0 l water (3.26 l for the ID 125) in preparation for the hydrostatic displacement step below. [The upper chamber is not inside at this point.]

• To prepare the electrofocusing gel solution, measure the following into a 50 ml lyophilization flask:

	by weight	by volume
Urea	8.74 g	
30% acrylamide with 1.8% bis (see recipe section)	1.72 g	1.68 ml
NP-40 detergent (20% in water)	1.64 g	1.62 ml
Ampholytes	0.89 g	0.80 ml
Glass-distilled water	5.16 g	5.17 ml

Dissolution of urea is an endothermic process, and is aided by warming the flask and its contents. Do this by partially immersing the flask in warm water or heating briefly (and very carefully) in a microwave. Do not heat much above room temperature! Overheating can partially carbamylate the basic ampholytes and alter the pH gradient, as well as generate potentially reactive compounds. You may degas the solution briefly on a lyophilizer to remove dissolved gas. Oxygen inhibits polymerization, and other gases can come out of solution during and after polymerization to form bubbles between the gel and the glass tube. Don't degas too long, or the urea will come out of the solution again due to evaporative cooling of the solution. If this happens, warm the sample slightly until the urea goes back into solution.

NP-40 is used as a diluted stock solution (20%) because the undiluted material is quite viscous and therefore hard to pipette accurately or mix rapidly. Add the NP-40 detergent after degassing (so as to avoid foaming).

• Then add the following carefully and quickly (polymerization starts the moment these two ingredients hit the solution):

10% ammonium persulfate in water (initiator)	100 µl
10% TEMED in water (N,N-tetramethyl ethylenediamine: catalyst)	100 µl

The indicated amount of TEMED is typical: experiment with a range to allow for variation in other reagents and for temperature variations in the room. The polymerization proceeds faster as the temperature increases and vice versa. In a hot laboratory, use less TEMED.

• Mix the solution carefully but rapidly, then pour it into the lucite gel-casting boat (Figure 5.18.3).

• Since the upper buffer chamber of the ID 125 does not have support legs, hang it outside the unit from the side without notches and fill the casting boat from the front. A channel connects the two sides of the casting boat so that both sides fill simultaneously.

• Slowly lower the entire assembly carefully into the large vessel containing an appropriate volume of water (3.0 l if using the old tube gel unit and 3.26 l, the "Cast Fill" line, if working with the ID 125) to allow the acrylamide to rise evenly by displacement to the desired level in all tubes. Be particularly careful as the gel casting boat is submerged: do this very smoothly and slowly so that the gel mix is not turbulently stirred up by the water as the boat "sinks". Once the upper buffer chamber assembly is inside the lower buffer chamber, be sure the upper buffer chamber hooks are fully seated into the slots cut in the lower buffer chamber when working with the ID 125. Check the level of water in the lower buffer chamber; you may need to remove or add a little water to bring the gel level to the correct point about 5 mm below the base of the upper buffer chamber (Figure 5.18.4).

• Allow the gels to polymerize for at least 1 hour. A polymerization line should be visible just below the meniscus of gels in the tubes and in the boat.

5.5 Disassembly Following Polymerization

• Remove the entire assembly from the outer buffer chamber.

• Withdraw carefully the casting boat from the tubes preventing a vacuum from pulling gel from the tubes. To do so, with the assembly laying horizontally, remove one of the casting boat face plates while holding the boat with one hand underneath.

• Remove the loose extra gel from the ends of the tubes, cutting it with a razor blade. Place the excess acrylamide gel in the trash can and not in the sink (it is insoluble and can block plumbing).

• Repeat the process for the other side and then remove the middle part of the casting boat (be sure none of the tube gels is attached to the loose extra gel).

• The water from the lower buffer chamber is usable to make the anodic buffer. Simply add 3 ml (3.26 ml to "Run Fill" line if working with the ID 125) of 85% phosphoric acid, making 0.015M acid.

• Reinsert the upper chamber (with attached tubes) into the box containing the acid solution. Slide the upper chamber forward so that it fits under the front lip on the inside of the lower chamber box. This lip prevents the upper chamber from being lifted out by the electrical leads when you remove the lid later. Once again, be sure the upper buffer chamber hooks are fully seated into the slots cut in the lower buffer chamber when using the ID 125 (notice that this model has no lip on the inside of the lower buffer chamber).

• Check that the buffer level in the lower chamber is between the lower electrode and the bottom surface of the upper chamber (do not exceed the "Max Fill" line on the ID125).

5.6 Prefocusing

• Prepare CO_2-free upper buffer and place this in the upper chamber. The upper electrode solution is generally prepared as follows: 200 ml of glass-distilled water are degassed (if desired) and 0.4 ml 10M NaOH (or 0.21 ml of 50% NaOH) added. This makes 0.020M base. For the ID 125, 150 ml of water and 0.30 ml 10M NaOH (0.15ml of 50% NaOH) are recommended (the ID 125 upper buffer chamber holds slightly less).

• Remove the air remaining in the upper portions of the gel tubes using upper buffer and a 100 µl Hamilton syringe. To do this, fill the syringe with upper buffer, insert the needle in a tube (placing the tip near the surface of the liquid remaining over the gel) and gently expel the buffer to displace the air out as a bubble. Be careful not to disturb the top of each gel. The urea solution that remains unpolymerized on top of the gel is left in place as a protective overlay. After debubbling all the tubes, look down into the tube ends from the top: you can see if any bubbles remain.

• Now place the lid over the banana plugs and press downward to make electrical contact.

• Prefocus the Iso set-up for 1 hour at 200 volts (Figure 5.18.5). This is a good time to assemble samples and fill out a run sheet indicating which sample is going into each tube (see section 16).

• After prefocusing, turn the voltage down to zero and switch off the power supply. Then remove the Iso lid by pulling it straight up.

5.7 Sample Loading

• Load sample into each tube you intend to run using a Hamilton or equivalent syringe (Figure 5.18.6). The 10 µl size syringe is usually best, though at higher sample volumes bigger sizes can be useful. If you have fewer than 20 samples, run some in duplicate; extra gels can be frozen and can prove useful in case of problems with later phases of the analysis. Sample volume can range from 1 or 2 µl up to a usable maximum of about 30 µl. A sample of 10 µl volume usually gives best results. Using sample preparation methods described above, a 2-10 µl sample gives a good Coomassie Blue-stainable pattern, and silver stain requires about 10-fold less. Record on the run sheet the volume actually loaded.

• For some samples, especially those that have been radiolabeled, it is often useful to add 2 µl of the creatine kinase (CPK) charge standards mix (**16**) (see recipe section) to each tube before adding the sample. These standards provide internal calibration for the focusing dimension. They can be conveniently applied using a Hamilton repeating microliter syringe. Similar standards are now also available commercially from Pharmacia and BDH.

5.8 Focusing

• Replace the lid and apply focusing voltage. For most samples, appropriate run times should be around 14,000 V*hr for 7" (18cm) gels and 30,000 V*hr for 10" (25 cm) gels (**17**). This is conveniently done overnight: for example, 17.5 hours at 800 volts for the 7" gels. Since some samples may require a different run length, it is best to do a focusing time

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16) Analytical techniques for cell fractions. XXIV. Isoelectric point standards for two-dimensional electrophoresis. Anderson, N.L. and Hickman, B.J., Anal. Biochem. 93: 312-320, 1979.

17) Focusing duration is generally given in a unit (V*Hr) obtained as the product of volts applied times hours run. Within limits, the time and voltage used are not critical as long as the correct V*Hr value is achieved. The V*Hr required typically varies as the square of gel length.

course to determine optimum running time for any new kind of sample (see section 5.14). If the danger of sample zone overload is particularly great, it may be useful to ramp the voltage up gradually.

5.9 Gel Unloading

• Turn the voltage down to zero, turn off the power supply and gently remove the Iso lid. Remove the Iso upper buffer chamber with the tubes (tilting it back so as to disengage from the retaining lip in the front of the outer chamber).

• Drain the upper buffer solution into the sink (provided it isn't radioactive; see section 5.16), and lay the Iso upper chamber on its back. Remove tubes one by one beginning with #1. (It is possible to unload tubes while they remain in the upper chamber; however, the tube being unloaded must be held to prevent its being forced out by the syringe.)

• Extrude the gels with the aid of water pressure provided by a small syringe (Figure 5.18.7). Hold the tube in one hand and the syringe in the other so as to inject water into what was the top end of the Iso tube (where sample was applied). Hold the tube and the syringe in opposite fists, using your thumb to press on the syringe plunger. To make a suitable syringe gadget, cut the large end off a "yellow"-size Eppendorf-type micropipette tip such that it will fit tightly on the Luer nipple of a 1 ml disposable plastic syringe. The syringe is filled with pure water, the tip inserted into the top end of a gel tube and the gel extruded by means of water pressure generated by pressing downwards on the syringe plunger. Pressure should be the minimum required to make the gel move, and should be as steady as possible to avoid making the gel move too fast. If the gel is extruded rapidly, it will tear. (Needless to say, a torn or broken Iso gel produces very misleading results.) The pressure required diminishes as the gel comes out due to the decrease in contact area between gel and glass tube. The objective is a perfectly smooth, slow extrusion process, taking about 10 seconds per gel. Be sure to avoid air bubbles trapped in the syringe; these will be compressed at the beginning, when high pressure is required, and will then expand again near the end of extrusion causing the gel to come out too fast.

• If available, an old HPLC pump can be connected to the tip to deliver even flow and adequate pressure to extrude the gels.

5.10 Storage

Even though unloading Iso gels directly (and immediately) onto the Dalt slabs is highly recommended, it is possible to extrude the Iso gels into individual numbered vials containing equilibration buffer (2 ml per vial) and freeze them. Either apply the Iso gels to Dalt slabs immediately, or freeze them at -70°C: the proteins will diffuse if the gels are kept too long at room temperature. If the gels are to be frozen, freeze them rapidly (e.g., in a dry ice bath) to prevent extraction of water from the gel during freezing. If freezing is done more slowly (for instance by placing the equilibration vials in a box in a -80°C freezer), the gels may be thinner upon thawing.

5.11 Cleaning the Iso Apparatus and Tubes

When the run is finished and all the tubes unloaded, clean the tubes as described above in section 5.2.1. If gel or protein dries in a tube, one has a problem analogous to egg dried on a dinner plate: it can be difficult to achieve complete cleaning later. To avoid this problem, keep tubes wet until a thorough cleaning is possible.

If something remains on the inside of a tube, it can often be removed by drawing an "Oral B SuperFloss" (a type of dental floss with an expanded, spongy region) through the tube, and then cleaning again with appropriate liquid cleaner.

Always rinse the Iso apparatus with plenty of very pure water after cleaning and allow it to dry in air. Store in a dust-free location.

5.12 BASO/NEPHGE Gels for Basic Proteins

Very basic proteins streak off the right side of the gel in the conventional O'Farrell-type 2-D system. For some reason (which should be understood but isn't fully), the basic end of an ampholyte-generated pH gradient is not stable and gradually slides off the gel towards the cathode (a process called "cathodic drift"). Therefore, alternative methods have been devised for separating basic proteins, involving use of nonequilibrium conditions (NEPHGE - non-equilibrium pH gradient electrophoresis; **18**)

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18) High-resolution two-dimensional electrophoresis of basic as well as acidic proteins. O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H., Cell 12, 1133-1142, 1977.

rather than true focusing. The basic protein 2-D patterns generated by these methods will not exactly superimpose upon IEF/SDS patterns in the overlap region where they share proteins.

This technique has been modified for use with the ISO-DALT® system (**19**) and the resulting gels are generally referred to as "BASO" gels. Samples are prepared as for IEF's, except that an acidic solubilization solution is used (see recipe section). The first-dimension gels are cast in the Iso apparatus using the same recipes as used for conventional IEF's with the one exception that the ampholyte used is only wide range.

After the gels have polymerized, the lower tank reservoir is filled with 3 l (or 3.26 l if operating the ID 125) of 20mM NaOH (degassed if desired). BASO gels are not prefocused, and the samples are applied with a Hamilton syringe directly to the upper gel surface. To protect the proteins from the acids used in the upper reservoir, samples may be overlaid with 4M urea (degassed), filling the remainder of the focusing gel tube. The upper reservoir is then filled with 200 ml (150 ml for ID 125 model) of 10mM H_3PO_4. For some samples, such as basic protein from seeds, it is not necessary to overlay the sample with urea; the upper buffer is simply injected into each tube to debubble the tube before the sample is loaded.

Note that the lower reservoir now contains the catholyte and the upper reservoir the anolyte, the reverse of the Iso system. The electrical leads are also reversed at the power supply (the black wire goes to the red terminal, etc.). To maintain a safe system, do not alter connections on the Iso lid or try to use the lid in a reversed orientation. Remember to switch the leads back to the conventional polarity if you share equipment with someone else, since the reversed polarity will ruin conventional Iso runs started in this configuration.

The run is started at 400 volts for 1 hour and then turned up to 800 volts for 4-5 hours, stopping the run at about 4000 V*hr for most samples. It is, once again, advisable to do a time course (section 5.14). Since the method does not achieve even a pseudo-equilibrium, catching the pattern at the right time is even more important.

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19) Analytical techniques for cell fractions. XXVI. A two-dimensional electrophoretic analysis of basic proteins using phosphatidyl choline/urea solubilization. Willard, Karen E., Giometti, Carol S., Anderson, N. Leigh, O'Connor, Timothy E., and Anderson, Norman G., Anal. Biochem. 100, 289-298, 1979.

Following the run, the gels are extruded and loaded as usual on Dalt plates "backwards", preserving the conventional acid=left orientation.

5.13 ACIDO Gels for Very Acidic Proteins

Some samples (e.g., urine) contain very acidic proteins which can be lost from the acid end of a conventional IEF gel. Such proteins often appear to be highly sulfated. In such cases, a modified system is used that depends on a non-equilibrium approach as for the BASO above, but in the opposite direction: the ACIDO system. For example, the most acid urinary protein (MAUP; **20**) can be resolved through an ACIDO run into a micro-heterogeneous pattern of perhaps 50 spots, whereas on a conventional IEF gel only a smudge at the acid end would be visible.

Gels are prepared in the Iso apparatus as for conventional IEF, except that the gel recipe is modified as follows:

- instead of .80 ml of ampholyte, a mixture of 0.3 ml pH 2-11 Servalyte® (Serva) and 1.0 ml pH 2.5-4 Ampholine® (LKB) is used, and
- added water is reduced by 0.55 ml

Allow the gels to polymerize for 1 hour. Prepare lower chamber buffer with 4.2 ml concentrated H_2SO_4 added to 2.8 l water. Prepare upper buffer by degassing 40 ml water and adding 1 ml 2-11 Servalyte. The top buffer barely covers the tubes: upper buffer volume is kept small because the ampholyte is expensive. Prefocus for 1 hour at 200 volts. Load the gels and run for about 3600-4000 V*hr (e.g., 800 V for 5 hours or at 200 volts for 18-19 hours overnight). Unload and run in second dimension as for IEF/SDS.

5.14 Performing a Time Course

It is wise to run a systematic test to determine when the proteins of an appropriate prototype sample are best resolved. Despite the fact that the name of the IEF technique implies that proteins are "focused" and therefore should come to rest at an equilibrium position, the phenomenon of cathodic

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20) Proteins of human urine. III. Identification and two-dimensional electrophoretic map positions of some major urinary proteins. Edwards, Jesse J., Tollaksen, Sandra L. and Anderson, Norman G., Clin. Chem. 28: 941-948, 1982.

drift prevents the use of very long focusing times in ampholyte-based systems. With BASO and ACIDO systems, optimum separation is, of course, achieved long before an approach to equilibrium.

Thus it is important to find the time (really the number of volt-hours) at which the proteins are best separated. This is done by comparing a series of identical samples run in the first dimension for varying lengths of time. In a time-course involving conventional IEF, for example, you will see the proteins begin at the basic end, run (or in many cases streak out) towards the acid end, stop at appropriate positions (isoelectric points), and then start to move back towards the origin again (as a consequence of cathodic drift). Performing a time-course run at the beginning of an extended study will allow optimization of resolution and also provide a good basis for understanding variations in pattern that may arise later due to changes in run conditions.

A reasonable general approach is as follows:

- Starting with a good initial guess of the length of the run in V*hr (your "expected" value), compute a series of ten different V*hr values by subtracting 10%, 20%, 30% and 40%, and adding 10%, 20%, 30%, 40% and 50% to your estimate. The ten values then span from 60% to 150% of the expected value.

- Set up the Iso apparatus with 20 gels of the appropriate type and load all tubes with the same amount of the same sample.

- In late afternoon, begin to run at a voltage such that 60% of the expected V*hr will have been reached at a convenient time the following morning.

- Beginning the next morning, take off the first time point by turning off power to the Iso, gently removing the lid and then the upper buffer chamber with tubes. Slide two tubes (e.g., 1 and 11) up through their grommets so that the tops of these tubes are about 0.5 cm above the level of the upper buffer and leave them there until posterior unload onto Dalt slab gels. (This will eliminate the circulation of the electric current through these tubes and prevent leakage of upper buffer if their gels are removed).

- Extrusion and storage of these two gels may be done at this point. Leaving the tubes in place, carefully remove the gels by water pressure (as described in 5.9 above) and collect them in equilibration vials. Place a vial of equilibration solution under the end of the raised tube (while the Iso

stands on the bench, or hangs outside attached to the lower buffer chamber if working with the ID 125), and hold the tube during extrusion with one hand to prevent its being forced down while at the same time pressing the syringe against its top. Equilibrate and freeze the first dimension gels as described above.

• Return the Iso unit to the lower buffer chamber, replace the lid and restart the power. Increase the voltage for the remainder of the run to a level that ensures completion of the series (150% expected V*hr) by the end of the day.

• Continue to remove pairs of tubes (raising the appropriate tubes to cut the electric current or prevent buffer leakage) at the remaining time points.

• Run one or both sets of ten focusing gels on Dalt slabs and perform appropriate detection of the proteins. Choose the best-resolved pattern and adopt the appropriate optimized V*hr value.

5.15 Comparing Different Ampholytes

It is often advantageous to try a variety of ampholytes and ampholyte mixtures before proceeding with an extended investigation. We make use of the fact that the tubes can be removed from the Iso apparatus, allowing preparation of a series of gel compositions which can then be run together under the same conditions for comparison.

Besides using a prototypical sample of the type to be run routinely, it can also be useful to run a series of pI markers like the charge standards (see recipe section) to assess pH gradient smoothness. Many otherwise good ampholytes have expanded (flat) and contracted (steep) regions that can lead to problems in matching gels accurately by computer. These characteristics become apparent if charge standards are compared with the different ampholytes available (see Figure 5.18.8).

Among the wide-range ampholytes currently available, Pharmalytes® (Pharmacia) appear to give the smoothest pH gradient. However, this comes at the price of increased ampholyte-protein interaction (making it quite difficult to focus large acidic proteins, e.g., tubulin), and increased staining of ampholytes fixed in the Dalt gel (at the bottom basic corner). Ampholines® (LKB) are good but have several pronounced gaps in most 2-D use (the largest almost exactly at the pI of cellular actin), thereby

increasing the difficulty of matching gels accurately. Resolytes® (BDH) and Bio-Lytes® (BioRad) both represent good compromises in performance. Servalytes® (Serva) are particularly good for BASO gels.

• Begin by preparing a double recipe of focusing gel up to the point of TEMED and persulfate addition, but leaving out ampholytes (the double recipe makes subsequent pipetting and additions easier). Divide into 5 or 10 equal aliquots in small tubes, and add to the aliquots appropriate volumes of the different ampholytes under test (e.g., 0.15 ml wide-range ampholyte into one of 10 aliquots of the double recipe). Remember that basic ampholytes are usually packed at only one-half the concentration (20% w/v) of wide range ampholytes (40%), though you need the same final concentration.

• Take 20 clean Iso tubes and connect a 1 ml disposable syringe to each by a piece of plastic tubing. Mark all tubes at the same desired gel height.

• Add TEMED and persulfate (scaled appropriately: 20% usual volumes for the case of 10 aliquots from a double recipe) to one aliquot at a time. After addition of the polymerizers, place the free end of an Iso tube into the aliquot and use the syringe to draw up gel solution to the mark (repeating to obtain two gels of each composition).

• When the gels are polymerized, assemble the tubes into the Iso, taking care to record the composition in each. Load all gels with the same amount of the same sample and run for the expected required V*hr. Load on Dalt gels for the second dimension and finally, compare the 2-D patterns to choose the best ampholyte for further study.

5.16 Radioactive Samples

In addition to taking the extra care normally associated with the handling of radioactive samples, you should measure the total radioactivity present in the upper and lower buffer after the run. If either exceeds the safe sink-disposal limits in your location, then the appropriate electrode buffer(s) must be placed in a liquid radioactive waste container for approved disposal.

5.17 Condensed Iso Instructions

(**Note:** Specifications for the ID 125 model in **bold**)

• Fit the gel casting boat around the two rows of glass tubes while the screws holding the boat together are loose. **(Fit the gel casting boat around the two rows of glass tubes while the upper buffer chamber is upside down.)**

• Tighten the boat assembly screws **(fasten face plates to the casting boat with "c"-section clamps)** to both seal the boat and grasp the tubes.

• Fill the empty lower (outer) chamber of the Iso system with approximately 3.0 l pure water **(3.26 l, e.g., to "Cast Fill" line)**.

• Measure the following into a 50 ml lyophilization flask:

Urea	8.74 g
Ampholytes	0.80 ml
30% acrylamide with 1.8% bis (see recipe section)	1.68 ml
Glass-distilled water	5.17 ml
NP-40 detergent (20% in water)	1.62 ml

• Degas (if desired) gently before the NP-40 addition.

• Then add the following carefully and quickly (polymerization starts the moment these two ingredients hit the solution):

10% ammonium persulfate in water (initiator)	100 µl
10% TEMED in water (N,N-tetramethyl ethylenediamine: catalyst)	100 µl

• Mix the solution carefully, then pour it into the lucite gel-casting boat.

• Lower the entire assembly carefully into the large vessel containing an appropriate volume of water to allow the acrylamide to rise evenly by displacement to the desired level in all tubes. **(Be sure the upper buffer chamber hooks are fully seated in the slots cut in the lower buffer chamber.)**

• Allow the gels to polymerize for at least 1 hour.

• Remove the entire assembly from the outer buffer chamber.

• Loosen the screws **("c"-section clamps)** of the boat and withdraw one of its face plates while holding the boat with one hand.

• Remove the loose extra gel from the ends of the tubes and repeat the operation with the remaining casting boat plate.

• Add 3 ml of 85% phosphoric acid **(3.26 ml; to "Run Fill" line)** to the water in the lower buffer chamber.

• Reinsert the upper chamber (with attached tubes) into the box containing the acid solution.

• Place 200 ml of glass-distilled water **(150 ml)** and 0.4 ml 10M NaOH **(0.3 ml)** in the upper chamber.

• Remove the air remaining in the upper portions of the gel tubes using upper buffer and a Hamilton syringe.

• Slide the upper chamber forward so that it fits under the front lip on the inside of the lower chamber box. **(Be sure the upper chamber hooks are fully seated into the slots cut in the lower chamber.)**

• Prefocus the Iso set-up for 1 hour at 200 volts.

• Turn the voltage down to zero and switch off the power supply. Then remove the Iso lid by pulling it straight up.

• Load sample into each tube you intend to run using a Hamilton syringe.

• Initiate the first dimension focusing run. For most samples, appropriate run times should be 14,000 V*hr for 7" (18 cm) gels and about 30,000 V*hr for 10" (25 cm) gels.

- When the run is completed, turn the voltage down to zero, turn off the power supply and gently remove the Iso lid. Remove the Iso upper buffer chamber with the tubes.

- Drain the upper buffer solution into the sink and lay the Iso upper chamber on its back. Remove tubes one by one beginning with #1, loading them directly onto Dalt slabs.

- For storage, extrude each gel into its own numbered vial containing equilibration buffer (2 ml in each tube).

5.18 Iso Figures

The following figures illustrate the use of the Iso apparatus.

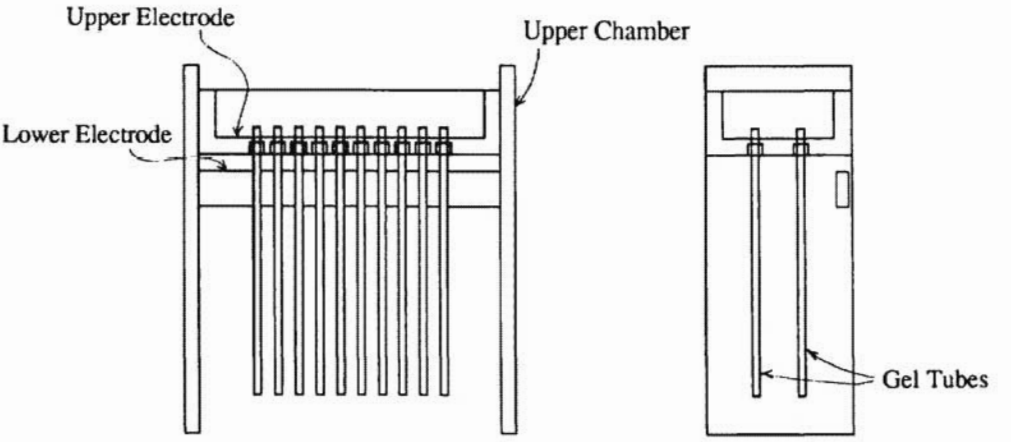


Figure 5.18.1: Iso upper chamber standing with tubes in position. Upper chamber of Hoefer's ID125 is supported by hooks rather than legs.
FRONT and SIDE VIEWS

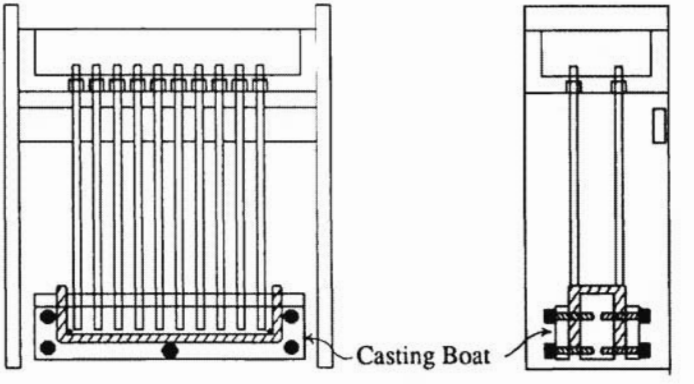


Figure 5.18.2: Iso with gel casting boat mounted on tubes. In the ID 125, plastic clamps replace screws holding center and two outer face plates of the casting boat together, gripping the tubes.
FRONT and SIDE VIEWS

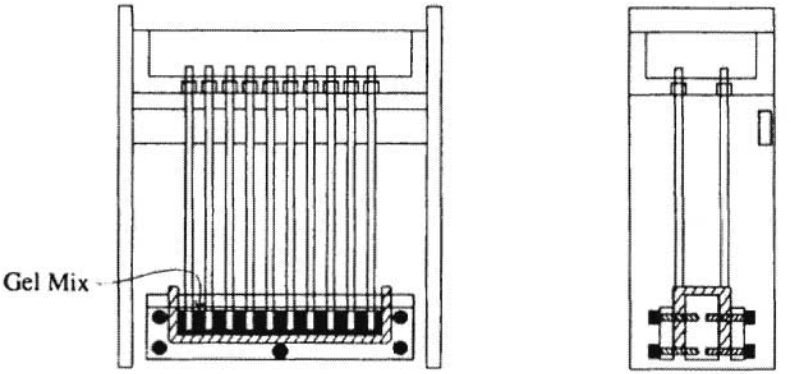


Figure 5.18.3: Iso with gel mix loaded into casting boat
FRONT AND SIDE VIEWS

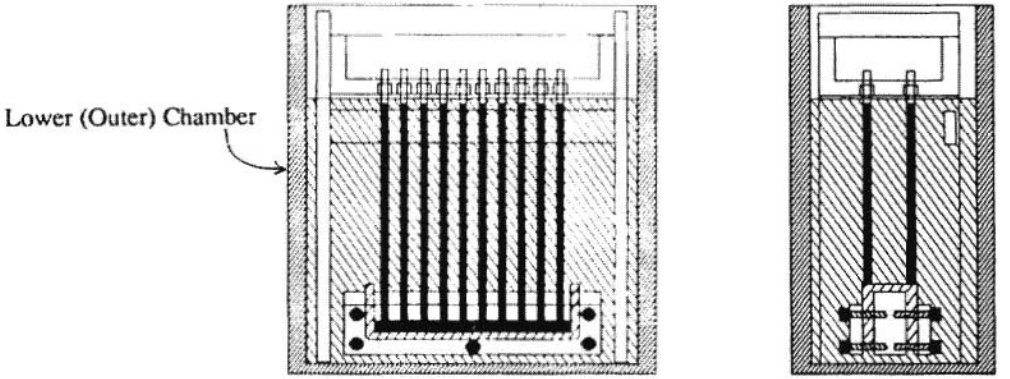


Figure 5.18.4: Iso immersed in lower (outer) chamber: Gel mix is displaced up into gel tubes by hydrostatic pressure.

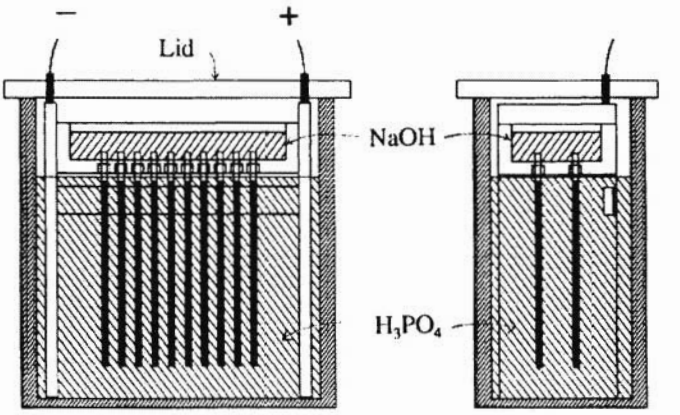


Figure 5.18.5: Iso apparatus in running configuration: Gels polymerized, casting boat removed and electrode buffers loaded.

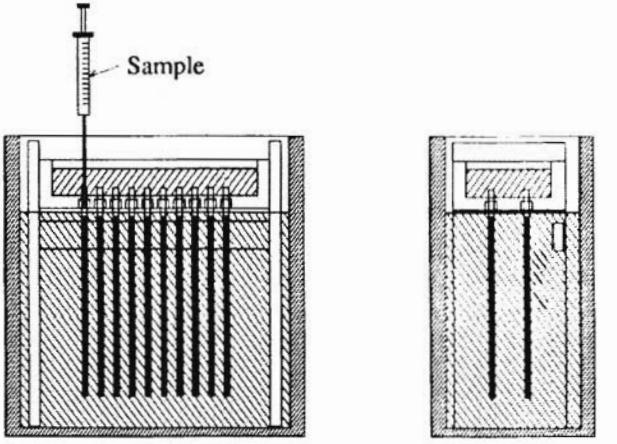


Figure 5.18.6: Sample loading onto Iso tube gels.

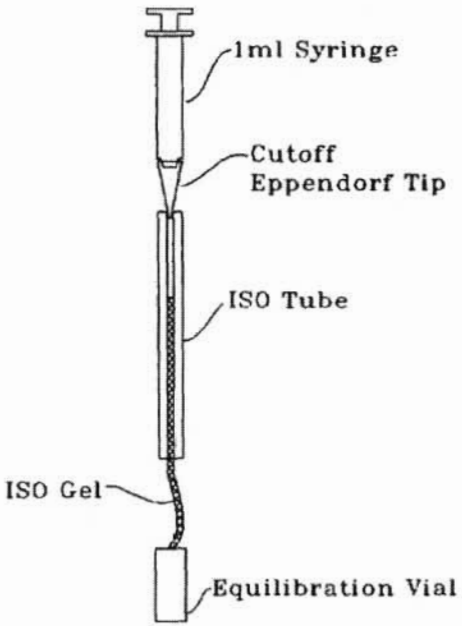
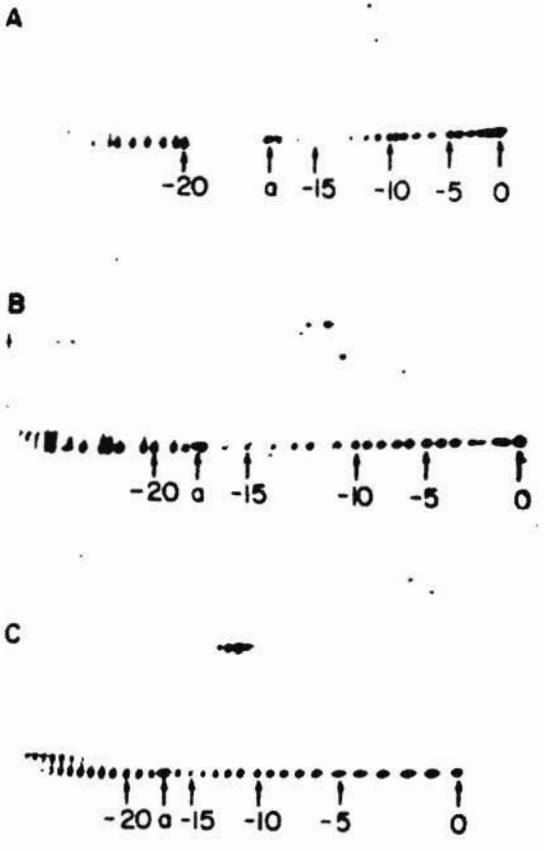


Figure 5.18.7: Unloading Iso gel from tube into equilibration vial using water pressure from syringe.



Sections from two-dimensional gels of human peripheral lymphocytes co-electrophoresed with CK carbamylation train standards and stained with Coomassie Blue so only a few lymphocyte spots appear to show differences in separation using different ampholytes. (A) LKB pH 3.5–10; (B) Servalyt ISO-DALT grade pH 3–10; (C) Pharmalyte pH 3–10. In all cases actin (a) is located at charge -17.5.

Figure 5.18.8: Use of CPK charge standards in the comparison of different ampholytes for the first dimension Iso separation (figure reproduced from **21**).

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21) The use of carbamylated charge standards for testing batches of ampholytes used in two-dimensional electrophoresis. Tollaksen, Sandra L., Edwards, Jesse J., and Anderson, Norman G. Electrophoresis 2: 155-160, 1981.

5.19 Iso Notes

Iso Notes

6 Dalt: the Second Dimension

The second dimension separation of the O'Farrell 2-D procedure involves slab gel electrophoresis in the presence of the detergent sodium dodecyl sulfate (SDS). SDS interacts with proteins to dissociate subunits, "unrolling" the polypeptide chains and coating them to yield flexible rods whose lengths are effectively proportional to polypeptide molecular mass. SDS carries a strong negative charge, and hence SDS-protein complexes are uniformly negative on a charge-per-polypeptide-chain-length basis. An electrophoretic separation of these complexes carried out in a microporous gel (such as acrylamide) therefore operates effectively as a sieving process, separating according to rod length (proportional to molecular mass). Large molecules move slowly and small molecules more rapidly.

In most SDS systems, an additional electrophoretic phenomenon is employed to increase resolution: "stacking" of the sample molecules just as the separation begins. This is achieved as a result of having different negative buffer ions in the gel slab and in the electrode buffer (in this case, chloride in the gels and glycine in the buffer). All the SDS-protein complexes have a mobility between those of chloride and glycine at the "stacking" pH (~6.8), causing the sample molecules to be compressed (that is, stacked) into a very thin starting zone (the stacking front). Once the protein-SDS complexes enter the separating gel (at about pH 8.8), "unstacking" occurs with the pH-induced change in glycine's net charge. This allows each protein to migrate at a rate appropriate to its size.

In the ISO-DALT® system, the sample zone (the Iso gel) is small enough to serve as its own stacking region when, following equilibration, its pH is set correctly for stacking and SDS is introduced. Therefore Dalt slab gels typically use no separate stacking gel (**22**).

6.1 Apparatus

The Dalt apparatus was designed to allow a reasonably large batch of slab gels to be run in a compact unit under identical conditions. Since it is

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22) It is, in fact, possible to run relatively good 2-D gels in this system using no stacking at all. If special circumstances (e.g., pH sensitivity) require avoidance of a Tris/glycine-type system, a homogeneous buffer (same in both gel and tank buffer) can be used with moderately good results.

intended primarily for 2-D work, it makes use of the fact that the sample is solid by electrophoresing the gel in a "sideways" orientation, with the Iso standing vertically rather than lying horizontally as in a conventional slab apparatus. The approach is also applicable to 1-D separations where the sample can be made up in agarose, or made solid by some other means. Because of the small sample zone used (generally the size of the Iso gel rod), a stacking gel is usually not necessary in this system.

6.1.1 Gel Cassettes

The slab gel cassettes are made in the shape of books: the two glass plates are held together by a strip of silicone rubber glued with RTV along one edge. The glass spacers (1.5 or 1.0 mm) are glued in position, also with RTV. The result is a cassette which is assembled simply by closing the book; there are no parts that disassemble like classical multi-part gel plate systems. Gels are removed by opening the book after the run and lifting out the slab. The cassette is easily cleaned as a unit, and can be stood on its side on a flat surface (in the open position, V-shaped) to dry. The cassettes are dishwasher-safe.

Cassettes are available in the original 7"x7" size (giving a gel approximately 6.5"x6.5", or 42.25 square inches), or the newer, larger 8"x10" size (giving a gel of about 7.5"x9.5", or 71.25 square inches). The larger gels offer almost 70% greater separating area and can make the best use of the standard 8"x10" X-ray film, but are a bit less convenient to handle. LSB's Dalt electrophoresis tanks will accommodate either size of cassette.

6.1.2 Casting Box

Casting slab gels as a batch offers the best reproducibility available in manually operated equipment; however, casting with such volumes of acrylamide raises technical issues unfamiliar to many gel users. First, acrylamide solutions in the 10-20 %T range shrink upon polymerization by about 2%. This means that a batch of 22 gels 8"x10" will contract by about 40 ml (.02 x 2000 ml) during polymerization; special steps must be taken to compensate for this loss. Second, because of the exothermic nature of acrylamide polymerization, a gradient must be made to gel from the top downwards; otherwise, solution gelling first at the bottom would rise due to thermal convection, upsetting the gradient. Since this involves a lower

acrylamide concentration (at the top) polymerizing before a higher concentration (at the bottom of the gradient), reverse gradients of persulfate and TEMED are required. Third, it is desirable to provide a method of clearing gelling acrylamide out of the tubes that bring the gradient to the box, and from any recessed areas of the box itself, before polymerization takes place. Otherwise the user may have to dig acrylamide gel out of hard-to-reach places.

The design of a multi-gel casting box must therefore take into account the need to cast a reproducible density gradient, let it polymerize top-down, displace acrylamide from the feed lines and box entry port, and also allow for shrinkage of the acrylamide by permitting entry of displacing fluid from the bottom. The Dalt system satisfies these requirements.

The Dalt casting boxes (7"x7" and 8"x10" sizes) hold 22 gel cassettes (so as to include spares in the batch). Loading and unloading cassettes from the box is made easier by a removable front plate. A gradient is introduced from the bottom of the box. The gradient stream coming from the gradient maker is directed from the delivery tube into a grommet in the floor of a side chamber filled with dense displacing solution. While the feed tube is in place, gradient is delivered directly into the casting box. When the gradient delivery is finished (and before polymerization begins), the feed tube is removed, allowing dense displacing solution to run partly into the box (clearing the delivery tubes of gelling solution). As polymerization proceeds, additional displacing solution is drawn into the box at the bottom to make up for acrylamide shrinkage (see Figures 6.13.2-6.13.10).

6.1.3 Gel Numbers

To allow positive identification of gels, it has proven to be very convenient to label each slab through the incorporation of a small label typed onto thin filter paper. A carbon ribbon, photocopier or laser printer should be used if you make these yourself, since many typewriter inks are electrophoresed off paper during an SDS electrophoresis run.

A variety of numbering schemes are possible. In our experience the easiest uses three parts as follows:

- A CAPITAL letter identifying an investigator or an extended gel series.

• A two or three digit serial number identifying the slab gel batch.

• A lower-case letter identifying a gel in the batch. Since a maximum of 22 gels can be made in a batch, the letters a-v are used.

The resulting numbers, of the form A63a, A63b...., etc., provide a useful system for keeping track of and cross-indexing experiments and gel production.

6.1.4 Gradient Makers: Gravity, Pump-Assisted Gravity and Computer-Controlled

The Gravity Gradient Maker is a simple, gravity feed unit having two chambers defined by a silicone rubber gasket clamped between two acrylic plates. The chambers are separated by a movable "tongue" whose shape defines directly the shape of the gradient obtained.

• You will need to set the shape of the tongue to suit the gradient you want. (A straight tongue gives a linear gradient.) A simple approach is to fill the gradient with water to the volume necessary to cast the size gels you will use. Note this height, empty the device, and then loosen the clamping screws so that you can move the tongue. Make the tongue linear, with its upper end almost touching the left wall at the height determined. Bend the remainder of the tongue to the right so as to make a funnel for introducing heavy solution into the left side (see Figure 6.13.3).

• Liquid flows leaving the two chambers are joined in a Y-connector and then thoroughly mixed by a simple "bow-tie" in-line pipe mixer having no moving parts.

• Three simple pinch clamps are used to control the flow at the exits from the Light (1) and Heavy (2) chambers, and after the mixer (3) to control flow into the casting box (see Figure 6.13.3).

A pump-assisted version of this gradient maker, the ID 350, is available through Hoefer. It differs from the standard gradient maker in that a peristaltic pump is connected between the gradient maker and casting box (see Figure 6.13.4). Its use ensures even flow of the gel solution into the casting box.

The Computer-Controlled Gradient Maker (Angelique™) is a more sophisticated device that saves effort and improves reproducibility. A complete description of Angelique is provided in the following chapter.

6.1.5 Equalizer™

The flatness of the top gel surface is a major determinant of SDS slab gel quality and resolution. Imperfect gel tops can lead to irreproducible protein spot 2D-gel patterns. A convex or tilted slab top surface can give rise to double spots, as protein at the front and back gel surfaces starts moving at different points. The Equalizer is a device for applying the overlay very smoothly and slowly, and thereby avoiding any mixing of overlay and gel solution. The result is very flat slab gel tops.

The tool consists of a plastic plate with raised edges (forming a shallow box) in whose floor are mounted two rows of 11 tuberculin syringes (to overlay a batch of 22 gels at one time) spaced so as to match the distances between plates in the assembled casting box. The plate has two small legs to hold the Equalizer on the top of the casting box. The syringes (1 ml disposable plastic tuberculin syringes) are inserted in the platform vertically with blunt-ended needles projecting downward into the cavities of the Dalt cassettes. The syringe plungers have been removed so overlay solution can run straight through the syringe and out slowly through the needle. The distance between syringes and their heights with respect to the platform are set so as to have one needle in each cassette.

The overlay solution consists of sec-butanol saturated with Dalt gel buffer. The Equalizer provides the same amount of overlay solution to each gel (1 ml). After loading the Equalizer with buffer-saturated sec-butanol, the overlay process proceeds automatically.

6.1.6 Dalt Tank

The Dalt tank offers a compact, convenient solution to the problem of running large numbers of slab gels reproducibly. Gels are slid into the tank from the top, much in the way that printed circuit cards are inserted into slots in a computer.

The tank is divided into three chambers by two removable barrier combs. Each barrier is made up of an assembly of silicone rubber flap seals. These seals provide a good barrier to electrical current leaking around the gel cassette, causing most current to flow through the gel. There is, however, some leakage current, though this has no effect on system operation. The seals are not designed to be liquid-tight: this is why all three chambers are filled to the same height with the same SDS electrode buffer.

The left and right chambers contain platinum wire electrodes (cathode and anode, respectively) and the center chamber provides cooling via circulating buffer and a tube-type heat exchanger. Since the gel cassettes are almost entirely exposed to the buffer in the center chamber (only their ends protrude into the side chambers), cooling of center chamber buffer provides excellent temperature control of the gel slabs during the run.

Center chamber buffer circulation is provided by a magnetically-coupled centrifugal pump (mounted on the back of the Dalt tank) connected to input and output "flutes" in the tank by a network of tubing. Never lift or move the tank by taking hold of the tubing at the back of the tank. It's only plumbing, not a handle. Buffer is taken into the pump through the middle of the three flutes running along the length of the tank's center chamber, and expelled back into the tank through the left and right flutes. The holes in the output flutes are aligned so as to generate maximum circulation over both the cassettes and the heat exchanger on the floor of the tank's center chamber (beneath the flutes). As described in 6.2, the pump should only be run when the tank is filled with liquid (otherwise damage to the pump can result).

The cooling heat exchanger (the ceramic tubing on the floor below the flutes) is quite robust, but can be broken by a dropping a heavy, sharp object into the tank. Take care to avoid this: a broken heat exchanger can let coolant leak into the tank or buffer out of the tank. A run would be ruined either way and an overflow situation made possible.

The entire tank is filled with SDS electrode buffer to a level determined by the cassette size used. Any number of gels up to the tank maximum can be run; empty slots seal themselves and require no blank cassette. Buffer is removed from the tank using a liquid pump (see 6.1.11), since the filled tank is too heavy to empty directly. Between runs, the tank should be rinsed with water.

The Dalt tank is made in two sizes: 20- and 10-gel capacities. Both sizes accommodate either 7"x7" or 8"x10" gels through the use of a movable barrier comb.

6.1.7 Refrigerated Cooling Bath

Dalt tank cooling is provided by circulation of chilled liquid through a tube on the floor of the central chamber. A refrigerated cooling bath (e.g., Hoefer or Forma) is connected to the fittings at the back of the Dalt tank

and the bath's pump used to circulate a chilled water/antifreeze or water-/glycerol mixture through the coil. Be aware that antifreeze is usually toxic (containing ethylene glycol) and makes a fairly sticky mess if allowed to leak from the system. The bath is usually set at 0°C, which typically gives a Dalt tank running temperature of ~10°C. The Dalt tank's own pump circulates the central chamber buffer over the cooling coil and over the gel cassettes, ensuring good temperature control of the gels during the run.

6.1.8 Power Supply

For overnight or full-day runs, a conventional electrophoresis power supply delivering 500V at 400mA (e.g., Hoefer PS 500XT) is suitable. For multiple tanks or faster runs (<6Hr) a larger supply (i.e., Hewlett-Packard 6448B, 600V at 1.5A or Sorensen DCS 300-3.5, 300V at 3.5A) is desirable.

6.1.9 Lectern

A simple slab gel loading and unloading "lectern" is provided. For loading, the gel cassette is placed in a slightly "reclined" position with its top edge resting flush with a horizontal flat surface on which the Iso gel can be aligned for indirect-loading. For loading directly from the Iso tubes to the Dalt cassettes, extrude the Iso gel along one of the top edges of the cassette. The bottom edge of the cassette rests on two nylon screws which can be placed in different positions corresponding to the different cassette heights. The bottom pair of holes is for 8"x10" cassettes, the middle pair for 7"x7" cassettes, and the top pair for Hoefer SE400/600/700 size gel plates (Figure 6.14.2).

For unloading, a V-shaped platform allows the cassette to be pried open and the two walls rested in open position for removal of the gel slab (Figures 6.14.9 & 6.14.10).

6.1.10 Wedgies™

Wedgies hold first-dimension IEF gels in place during the second dimension run. The Iso gel is extruded directly from its tube into the space in the Dalt cassette, using a small amount of SDS equilibration buffer as lubricant. This direct transfer from tube to cassette reduces handling and ensures correct sequence of proteins in the event of Iso gel breakage. A Wedgie inserted into the space presses the Iso gel firmly onto the slab gel

and holds it in position during the run. As no sealant is used, no set-up time is required; the cassette can be immediately placed into the tank. The entire loading process takes half the time of earlier methods.

By eliminating the need to incubate Iso gels in equilibration buffer, Wedgies prevent the typical 10%-40% protein loss to buffer; all the focused protein in the gel is available to enter the Dalt slab cassette. Also, proteins electrophorese uniformly in the second dimension because the Iso gel is maintained in closer contact with the slab gel. In contrast to the molten agarose method, Iso gels are not exposed to heat that can impair gel quality. Low molecular mass proteins, that might otherwise diffuse during the loading process, remain in focused bands and are better resolved in the final gel pattern.

6.1.11 Additional Small Gadgets

- A small plastic spatula is useful for inserting Iso gels into the tops of the Dalt cassettes.

- A plastic tea strainer is very useful during Iso indirect-loading: the Iso is recovered from the equilibration solution by tipping the contents of the equilibration vial into the strainer, which is then inverted onto the Dalt loading lectern to deliver its solid contents (the gel) for loading.

- Plastic-covered dishracks (Rubbermaid or equivalent) are useful for holding the Dalt gel cassettes during the loading process. Usually two 13-place racks are used per set of 22 gels.

- Melting of the agarose overlay is facilitated by a microwave oven.

- A short screwdriver is useful as a tool for prying open the gel cassettes after the Dalt run.

- A small self-priming liquid pump (such as the Cole-Parmer J-7041-00) is strongly recommended for removing buffer and rinse water from the Dalt tank. The pump can be set up using garden hose or Tygon tubing to deliver expelled buffer (assuming that it is not radioactive, etc.) directly to a lab drain.

- Pump for degassing solutions.

6.2 Loading the Dalt Tank with SDS Running Buffer

• Two or three hours before the beginning of a run, fill the tank to the appropriate height with purified water (de-ionized is often good enough, though fully purified is better). Always use water near room temperature: never fill the tank with liquid at a temperature very different from the tank's. For 7"x7" cassettes, the required liquid height is about 5" below the top of the tank, and for 8"x10" cassettes it is about 2" below the top of the tank. Because the Dalt tank has its own circulating pump, the tank buffer is easily made within the tank itself.

• It is necessary to fill the pump with water before turning it on. The pump is centrifugal and thus not self-priming: if it is run dry it can be damaged. Turn on the pump after the tank is filled; it should begin by blowing some bubbles through the circulating flutes in the tank and then establish a vigorous circulating action in the tank. If the pump doesn't "catch" and no circulation is observed, quickly turn off the pump (you have an airlock in the pump), wait a moment for the air to bubble out through the small "bypass" tube coming up from the pump's outlet and entering the back of the tank about halfway up, then restart the pump. You may have to do this a few times. Once the pump is circulating, leave it on.

• Weigh out the correct SDS running buffer mixture for your gel and tank size (see recipe section) and add the dry powder directly into the center chamber, distributing it evenly from front to back. The pump will circulate the contents enough so as to dissolve the solids in about 1-2 hours (some will lie on the bottom for awhile, but this gradually disappears). It is convenient in practice to make a number of these buffer packets in 1 l plastic beakers and have them on hand.

• Once the buffer is dissolved, make sure to raise the two barrier combs (at the same time) so as to mix the total Dalt tank contents with the dissolved contents of the center chamber. The buffer is now the same in all three chambers and ready to use.

• Make a note on a label on the tank lid when you change the buffer. Although it is possible to use each tank of buffer for 2-3 runs (by mixing the center and cathode [left] chambers between runs), it is better to change the buffer before every run: fresh buffer ensures better results. When a tank is not going to be used for several days, pump out the buffer and rinse the

tank with water, allowing the tank to sit empty. This procedure will eliminate growth of bacteria, pseudomonas, etc., which can occur in old buffer. Such contamination can give rise to "mysterious" protein bands across a whole batch of gels.

•Place the barrier combs in the correct slots to accommodate the size of gel to be run (8"x10" or 7"x7" cassettes).

6.3 Preparing Slab Gels

The casting of slab gels for the second dimension separation by SDS electrophoresis is an operation that requires planning and care. The procedures and recipes described below are appropriate for making a full set of 22 gels. The recipes given (section 6.15) assume the gels will contain SDS when they are made: since SDS is introduced with the stacking front during electrophoresis, it is not absolutely necessary for it to be present in the gel beforehand. If SDS is not required in the gel, add the equal volume of water. Recipes are provided for both weight-weight (preferred) and volumetric preparation of gel solutions.

6.3.1 Loading the Casting Box

• Plan to set up the casting box in some tray, or other container, that can act as a catch basin for any liquid overflowing the box or emptying out of it when it's opened after the casting operation. It is often convenient to set the box up on a plastic or rubber kitchen drainboard one side of which empties into a sink. Check that the box is level.

•Stack 22 cassettes into the gel casting box with all hinge strips vertical and aligned on the side of the box opposite the feed tube, interspersed with plastic sheets (see below) and a couple of appropriately-sized sheets of "bubble-pack" material (Figures 6.13.1 and 6.13.2). The bubble-pack takes up any remaining space in the box and holds the cassettes gently but firmly in position. Cast 22 cassettes for a 20 sample run in order to allow for the possibility of one or two imperfect gels.

Sheets of plastic (e.g., 0.010" thick polycarbonate sheet) interspersed between the cassettes can make it easier to remove the cassettes after polymerization. This material is easily obtained and can be cut with scissors to the appropriate size (just slightly smaller than the size of cassette to be used). Plastic sheet may also allow improved gel quality when high %T

gels are cast: the cassette walls can bow inward slightly adjusting to the gel shrinkage on polymerization if plastic is present to prevent their adhering tightly together.

• Put the removable front plate of the casting box in place and screw on the knurled stainless steel finger nuts. Tighten these hand tight (not over-tight). Be sure that the box is not so full as to prevent the front plate from sealing tightly against the box (i.e., see that the O-ring gasket is sealing). The use of the bubble-pack interspersed with the cassettes allows some compression of the stack of cassettes as the front of the box is put on.

• Prepare a set of gel labels on filter paper. Cut the labels apart (leaving little excess around the characters), and place them in order in front of the gel casting box. Then, taking care to keep track of which cassette will be numbered next, drop the numbers in order into the cassettes (drop them into the side opposite the gradient inlet port). The numbers will fall to the floor of the casting box but will remain in the respective cassettes as the gradient is introduced, ultimately becoming polymerized into the gels themselves.

• Insert the end of the gradient feed tube (the tube coming from the gradient maker) into the grommet in the floor of the side chamber of the casting box (Figure 6.13.3 and 6.13.4). The feed tube must be snugly in place so that there is no leakage from the side chamber into the casting box at this point.

6.3.2 Forming a Gradient with the Gravity Gradient Maker

6.3.2.1 Checklist for Dalt Casting Procedure

• Be sure the entire gel casting system is clean, dry, and free from any polymerized acrylamide.

• Be sure that a sufficient volume of gel overlay solution is on hand. The overlay consists of buffer-saturated sec-butanol. About 22 ml is needed for a set of 22 cassettes.

• Be sure that the gradient-maker lines are clamped off. There are three clamps: one coming from each chamber and one after the mixer. Close all three.

• Make up the gel acrylamide solutions from the stock mixes as follows: weigh or otherwise measure the appropriate amounts of acrylamide stock solution, buffer D, SDS, pure water and glycerol (where required) for the Light and Heavy mixes selected (section 6.15). Note that the Light mix is always assumed to have a lower acrylamide concentration than the Heavy mix (i.e., the gradient increases in %T from top to bottom).

These solutions can be degassed, though this is not usually necessary.

• Add the appropriate volumes of 10% ammonium persulfate (not more than 1 week old) and 10% TEMED only when you are ready to pour the gradient, not before. Once these reagents are added, polymerization begins. The amounts of TEMED and persulfate are based on broad experience but nevertheless may not be correct in your lab on account of temperature, reagent quality, etc.. Therefore, perform a test before first using a new composition to check that your Light recipe polymerizes in about 10 minutes and your Heavy recipe in about 20 minutes.

6.3.2.2 Gradient Pouring Procedure

Instructions for Hoefer's ID 350 pump-assisted gradient maker appear in **bold.**

• Mix each solution when the ammonium persulfate and TEMED are added. You will have about 10 minutes to cast the gradient before the gels begin to solidify at the top. Work rapidly but carefully.

• Pour the Light solution into the right side of the gradient maker (the side whose chamber is wider at the top).

• Carefully open the clamp on the Light chamber exit tube (Clamp #1) and then very slowly open the Heavy chamber exit tube clamp (Clamp #2) so as to allow Light solution to fill the tube coming from the Light chamber all the way to the "Y" connector and back to the point at which the Heavy tube enters the Heavy chamber. The objective is to fill the entire tube with Light solution (no bubbles), but not to get Light solution into the Heavy chamber itself (Figure 6.13.5).

• Close both clamps again. All three clamps are now closed.

• Add the Heavy solution to the Heavy (left) chamber (the one that is wider at the bottom) until the liquid level reaches a point about 2 cm below the level of Light solution in the adjacent chamber (Figure 6.13.6).

• Fill the side chamber with a dense displacing solution (50% glycerol or 35% sucrose) containing a little bromophenol blue. None should leak into the casting box because of the grommet seal and the gradient feed tube.

• Open the clamp after the mixer (Clamp #3), thus opening the feed tube to the casting box (**to the peristaltic pump**).

• Carefully open the clamp on the Light chamber exit tube (#1). Light solution will begin to flow through the feed tube and mixer towards the casting box. At this point a small amount of Light solution is allowed to enter the casting box. **(With the ID 350, open clamp #1 and turn on pump to bring a small amount of solution into the casting box.)**

• When the Light solution level in the gradient maker falls to a level about 1 cm above the level of the Heavy solution, open the Heavy chamber exit tube clamp (#2). The reason for opening this clamp while there is still a height difference lies in the density difference between Light and Heavy solutions: the two are in hydrostatic equilibrium with a height difference of about 1 cm assuming 9% Light and 18% Heavy. Other gradient compositions may require a different height difference. The objective is a smooth start to the Heavy outflow.

• Watch the gradient enter the casting box (Figure 6.13.7).

• **Note that the flow rate of the pump head on the ID 350 is approximately 2.8 ml per revolution and the drive capability 50-600 rpm, giving the pump a range of 140-1680 ml/min. Hoefer recommends a pump setting of 280 ml/min to cast 7"x7" gels; that is a volume of 1400 ml. For 8"x10" gels (a volume of 2200 ml), a setting of 440 ml/min is suggested. At this rate a box of gels can be cast in 5 minutes.**

• When the gradient maker is empty or the casting box is filled to within 1 cm from the top of the cassettes (whichever comes first). (**Turn off pump**), close the feed tube clamp (#3), remove the gradient feed tube and place its end in a waste receptacle for polymerizing acrylamide (a beaker is suitable). Reopen clamp #3 (**and restart pump**) to empty the gradient maker completely. Don't put unpolymerized acrylamide down the drain. As soon as the feed tube is removed, the dense blue displacing solution will run down the connecting tube to the box and just a bit will enter the box. The gradient is now in hydrostatic equilibrium in the box, ready to polymerize (Figure 6.13.8).

• After any excess polymerizing acrylamide has left the gradient maker (and been collected in a waste receptacle), place the feed tube in a larger waste vessel or a sink drain. Pour about a liter of water into each chamber of the gradient maker and open all clamps **(and pump)** to flush the system.

• Continue flushing about 2 l more water through the gradient maker and tubing.

6.3.3 Dalt Slab Gels Overlay

The objective is to overlay the gels very slowly so that overlay spreads evenly across the cassette with a minimum of mixing, thereby resulting in a smooth, flat gel top surface.

• Make sure the syringes and needles of the Equalizer are not clogged from last use. A 10 µl syringe or any fine wire can be useful for unclogging the Equalizer.

• Place the Equalizer on the top of the casting box, perpendicular to the cassettes, assigning one needle to each cassette. Avoid placing the Equalizer in the central part of the casting box (if any damage is done to the gels due to a malfunction of the apparatus, it is better to risk the extremes of the gels where none or a small number of spots are to be obtained).

• Immediately after removing the feed tube from the casting box and with the Equalizer in position, fill each syringe with 1 ml of buffer-saturated sec-butanol. This is easily done from a plastic bottle with a pin-point hole in the tip (such as that used for contact lens saline). Any overflow of sec-butanol will collect on the Equalizer platform so that only the desired amount of overlay solution goes to each cassette.

• Allow all the overlay solution to drain from the syringes onto the cassettes (about 2 minutes). Before removing the Equalizer from the casting box, check that all the syringes are empty. If a syringe is clogged, manually overlay that cassette by pipetting and dispensing, very smoothly, 1 ml of buffer-saturated sec-butanol onto the gel surface.

• Dispose of the excess of sec-butanol from the Equalizer in the sink. Cover the top of the casting box with plastic wrap and let the gels polymerize.

6.3.4 Polymerization

• Allow gels to polymerize for at least 1 hour. It is important for the polymerization to proceed from the top down. You can observe this through the front and sides of the casting box. The dense displacing solution (glycerol or sucrose) level will fall farther as the gel contracts upon polymerization (Figure 6.13.10).

6.3.5 Unloading the Casting Box

• Remove the front of the casting box. The dense displacing solution will leak out at this point into the tray or drainboard beneath the box.

• Carefully unload the cassettes from the box using a single-edged razor blade to separate the cassettes. Wash each cassette carefully with water to remove acrylamide adhered to the outer surfaces (a dish brush is useful here). As each cassette is washed, put it hinged side up in a dishrack standing in a plastic container with about 1/4 of an inch of tap water. The plastic container retains the draining excess liquid from the surface of the gel, and the water in the container helps to maintain the gels with enough humidity (a dry gel breaks and is useless for electrophoresis). Remove and clean the thin polycarbonate sheets if you've used these between the cassettes.

• Examine the gels at this time for air spaces, uneven top surfaces or other defects. Discard any unsatisfactory gel and replace it with one of the extra cassettes. If all are good, put the extras in appropriately diluted slab gel buffer at 4°C to use for an odd run or for those rare occasions when more than three plates are bad in a single batch. It is also possible to store extra gels by wrapping the cassettes individually in plastic wrap and putting them with a wet paper towel in a sealed plastic staining box.

6.4 Loading the First Dimension Gels onto the Dalt Slabs

Load Iso gels directly onto the Dalt slab gels as soon as possible: the exposed edges of the gels tend to dry out over a period of hours. Coordinate both Iso and Dalt runs in such way that the end of the Iso run coincides with the end of unloading the casting box. Aim to start the Dalt polymerization 1 hour and 30 minutes before the end of the Iso run (1 hour for gel polymerization and 30 minutes for unloading the cassettes from the casting box).

•Place the Dalt gels in the dishrack in alphabetical order (with respect to the lower case letter of the identification label) and with the sample-application surface of the slab up and the label readable from the front. Make sure the Iso #1 is loaded in slab "a", Iso #2 in "b", etc...

• Extrude the Iso gel from the tube along one of the top edges of the cassette. The acidic end should be on the left side.

•Introduce the Iso gel rod into the loading cassette slot. Use a plastic squeeze bottle (such as that used for contact lens saline) containing Iso equilibration buffer. Push the Iso gel gently with the bottle while squeezing out a little bit of buffer (which acts as a lubricant). Use a small amount of equilibration buffer (enough to cover the rod gel).

•Smooth the Iso rod gel into place with a small plastic spatula. Handling Iso's is an art requiring practice and a certain amount of manual dexterity. Do not touch the Iso with your fingers, as this can cause protein contamination. Before holding the gel with the Wedgie, the rod gel must be completely straight, centered, and without any air bubbles between the Iso and Dalt gel surfaces (bubbles interrupt the electric field and can cause distortion of the 2-D pattern).

•Place a Wedgie on the top of the Iso gel as shown in Figure 6.14.5. Be especially careful not to press the Wedgie between the Iso and Dalt gels. Loss of contact between the gels will result in loss of protein entering the second dimension and a distorted 2-D pattern. (The alternative method is to use 1.0 ml melted 0.5% agarose made up in Dalt tank buffer (see section 6.11 and recipe section) to seal the tube gel in place. Care must be taken not to overheat the agarose or to allow bubbles in the agarose overlay. Cassettes thus overlaid must sit 2-5 minutes to allow the agarose to solidify.)

•Note on the ISO-DALT run sheet (see section 16) the number of the Dalt gel onto which this Iso gel is being loaded.

•Load the remaining Iso's similarly.

• If the Iso samples are frozen, they should be removed from the freezer just before use (best results are obtained when a small number of gels - e.g., 4 - are removed from the freezer at a time, each one being quick-thawed just before loading). Do not touch the Iso with your fingers, as this can cause protein contamination (wear gloves). Place a Dalt gel in position on the front of the lectern (with the loading slot of the cassette up

and the gel label readable from the front). Pour the first Iso onto a small sieve (e.g., a plastic tea strainer), letting excess equilibration fluid drain through into a beaker below. Then pour out the Iso gel rod onto the top of the loading lectern, remembering to clean the top periodically to prevent cross-contamination. The Iso will have the appearance and consistency of clear blue spaghetti. Position the Iso gel so that it is extended along the loading ledge with the acid end to the left. Manipulate it gently using a narrow spatula (Figure 6.14.2). In general the acid end of an Iso will be rough (since it was cut with a razor blade when the Iso casting boat was removed); the basic end will be smooth (since it is the top surface, polymerized under air). If SDS is present in the sample, a bulb of SDS will be found focused at the acid end of the Iso gel.

6.5 Loading Cassettes into the Dalt Tank

• Carefully load the cassette immediately after inserting the Wedgie which holds the Iso gel in place. The cassette is correctly loaded in running orientation in the Dalt tank slot with the Wedgie vertical along the left side and the rubber cassette hinge along the bottom (Figures 6.14.6, 6.14.7). It is helpful to dip the hinge side of the cassette into the tank buffer first so as to lubricate it before insertion into the flap seals. Slide cassettes firmly to the bottom. Be careful, as the plates slip easily once your hands are immersed in tank buffer; use both hands.

• After the first cassette has been introduced into the tank, close the lid (Figure 6.14.8). Attach the electrodes to make proper electrical contact with the power supply, if they have not been left in position. Red (the right chamber electrode) goes to plus (+). Turn on the power supply to run the loaded cassettes at a low voltage (no more than 50 volts).

• As each subsequent Dalt gel is ready to load, turn off the power supply, open the tank and insert the cassette. Then close the tank and restart the power supply while you prepare the next cassette. Since the use of Wedgies reduces the loading time appreciably (it takes about 45 minutes to load 20 cassettes), the difference in volts × hours run between the first and last gels loaded is insignificant.

• Notice that the buffer level in the tank starts out low (achieving correct height when all the cassettes are loaded). Hence the electric current passes through only a part of each gel (electric current being conducted by the tank buffer). To maintain an appropriate buffer level in the tank while running at the loading voltage, introduce a heavy and inert object (like a

water-filled one-gallon plastic bottle) carefully into the center chamber. After half of the cassettes have been loaded, you can remove this "buffer level raiser" (the volume of the cassettes being loaded having increased the tank buffer level).

• Note the buffer level after all the cassettes are loaded in position; the level should be even with the uppermost spacer of the cassette and neither above the top of the cassette nor below the level of the top edge of the gel. If the level is too low or too high, adjust the liquid level to the correct height using added pure water or the removal pump (see 6.1.11).

6.6 Electrophoresis in the Dalt Tank

• Increase the voltage to the desired value on the power supply and run the gels until the blue tracking dye reaches the right side of the gel holder as seen through the front of the Dalt tank (the "bottom" of the gel). We recommend running the tanks slowly at about 100-150 volts (~10°C) overnight. However, a set of 20 7"x7" gels can be done in about 6 hours at maximum settings of 600 mA and 200 V. Depending on the gradient used, the pH of the buffers (and hence the conductivity), and the number and size of cassettes being run, run times can vary widely. Observe the progress of your first run and set up your schedule accordingly. The bromophenol blue tracking dye (which soaked into the Iso from the equilibration buffer) migrates just ahead of the smallest proteins: when the blue line reaches the end of the gel, the run is done.

6.7 Unloading the Dalt Cassettes

• When you are ready to remove the cassettes, turn off the power supply first. Open the Dalt tank lid only with the power off.

• Remove the cassettes carefully, one at a time, using the fingers of both hands. Rubber gloves can help give a good grip. The cassettes will be slippery because of the SDS detergent. Unload the cassettes into dishracks (ten per rack), placed in plastic containers with water (to decrease the risk of gels drying).

• Remove the Wedgies taking care not to tear the slab gels. Rinse the Wedgies with abundant warm water (the remaining SDS from the tank buffer is usually sufficient detergent to effect cleaning) and let them dry for the next run.

• Place a cassette on the unloading lectern and pry it open very carefully with a short flathead (i.e., not Phillips) screwdriver (Figure 6.14.9). This is best done by putting the screwdriver point into the top corner of the cassette away from the hinge and twisting it gently. Take care not to chip the glass of the cassette.

• When opening the cassette, make sure that the gel comes with one of the plates and is not sticking partly to both (the gel could tear).

• If the gel sticks to the plate with the spacers attached, run a single-edge razor blade down between gel and spacers to ensure that the gel is not sticking to either spacer. Always run a razor blade between gel and glass at the bottom of the gel.

• Carefully peel the gel from the glass (Figure 6.14.10), lifting it by the bottom (high %T end), and place it in a box of fixative solution. In case of a rip in the gel, remove the ripped section last; work toward the rip. Gels to be silver-stained should be handled only with gloves, since fingerprints will show after staining.

6.8 Cleaning Dalt Cassettes

• Soak the used cassettes in distilled water if you can't clean them immediately.

• To clean the cassettes, begin with a thorough rinsing in warm water, going over all surfaces with a Teflon "Tuffy" or plastic dish cleaning tool. (The SDS already on the plates is usually sufficient detergent to effect cleaning.)

• To avoid clogging the drain, use a fine, removable sink trap to catch shreds of polymerized acrylamide.

• Finally, rinse the cassettes with distilled water and air dry them in the open position on the lab bench or in a drying rack. Store the cassettes in a closed cabinet to keep them free from dust.

6.9 Constant Percentage (Non-Gradient) Slab Gels

Non-gradient gels are prepared in a manner very similar to that described for gradients (section 6.3), except that the gradient maker is not used (consult the following chapter if you are using Angelique). Instead, the feed tube (normally connected to the gradient maker below the in-line

mixer) is connected instead to a funnel held in a ring-stand at a level about 12 inches above the top of the casting box (Figure 6.13.11). The other end of the feed tube is placed, as before, in the grommet in the casting box side chamber. The box is loaded with cassettes, the front plate secured, the feed tube inserted in the side chamber grommet, and the side chamber loaded with heavy displacing solution as usual.

To cast gels, the gel solution (about 1400 ml for 7"x7" or 2200 ml for 8"x10") is poured slowly into the funnel, avoiding introduction of any bubbles into the feed tube line. The same recipes can generally be used (section 6.15) for single component gels as are used for Light components of gradients (you don't need the glycerol present in the Heavy mixes). Thus a Light recipe can be doubled in volume (or weight) to make a set of the appropriate sized gels.

Once the pouring is complete, the feed tube is removed from the side chamber grommet (as for gradients) and the displacing solution level in the side chamber falls.

The use of thin polycarbonate sheets between cassettes is particularly recommended in the case of non-gradient gels, since these do not polymerize from the top down and hence need to be able to contract inwards during gelation.

6.10 Stacking Gels (Optional)

A stacking gel (as used by Laemmli) is not often necessary because the Iso gel is so narrow and acts as its own stacker. However, for 1-D work in which a dilute sample causes use of a deeper sample zone, a stacker may be desired on top of the running gel. If so, proceed through the following steps after step 6.3.4 above.

- After the separating gels have polymerized, add separation gel buffer (buffer D diluted 1+3 with water) to each cassette to rinse the top of the gel.

- Pour off the surface fluid by tipping the casting box over carefully (the dense displacing solution will also pour out, so be ready for this). Blot the excess on top with a towel.

- Prepare a stacking gel mix:

Stacking gel buffer (see recipe section)	35 ml
30% Acrylamide, 0.8% bis (see recipe section)	10 ml
Water	25 ml
10% ammonium persulfate	1.4 ml
TEMED (10%)	500 µl

- Add 3 ml of the stacking gel preparation to each cassette.
- Use 1.0 ml 0.1% Photoflo™ in stacking gel buffer as an overlay for each cassette.
- Allow 15-20 minutes for polymerization.

6.11 Use of the Dalt System for High Volume 1-D Work

The Dalt apparatus can be used to run sets of reproducible slab gels for screening large numbers of samples by 1-D electrophoresis; or for production of "streak" slab gels (in which one sample is applied across the whole gel), for use in the production of Western blot strips (used in diagnostic kits) or for preparative isolation of a single SDS band.

In these cases the sample must be immobilized on the top of the slab, so that it stays in place when the gel is rotated for insertion into the Dalt tank. This is usually accomplished by mixing the sample with about 1.0 ml melted 0.5% agarose made up in Dalt tank buffer. The agarose is generally stored in a freezer in 100 ml portions and can be liquefied in a microwave oven or using a hotplate or waterbath. Be sure that the cap of the flask is loose or off during microwaving! Also note that solutions often superheat when they are microwaved so that they boil over when disturbed or when a pipette is introduced. Caution should be taken to direct the flask away from you when you start to use the agarose.

The sample is then applied onto the loading slot of the Dalt gel. Make sure that there are no bubbles in the agarose-sample on the cassettes. Allow 2-5 minutes for the agarose to solidify before moving the cassettes.

For multiple samples, each sample can be mixed with melted agarose and then pulled up in a pipette. When cooled, the sample can be extruded in the form of a rod, not unlike an Iso gel. Sections of one or more such

rods can be placed on the slab and sealed in place with normal Iso overlay agarose. The result of such a run is analogous to a typical vertical SDS gel run.

The major advantage of the Dalt system in 1-D work is the volume of analyses possible: in the single sample streak mode, the 20-place 8"x10" system yields 190" width of SDS separation (equivalent to a single slab almost 16 feet wide). In a multiple-sample screening mode, loading 25 samples per slab, 500 samples can be analyzed in one Dalt run.

6.12 Condensed Dalt Instructions (using the Gravity Gradient Maker)

Instructions specific to the use of Hoefer's pump-assisted gradient maker (the ID 350), appear in **bold**.

- Fill the Dalt tank to the appropriate height with purified water.
- Check to see that the coolant circulation is on.
- Turn the buffer circulating pump on once it is filled with water and check for flow.
- Weigh out the correct SDS running buffer mixture for this volume and add the dry powder directly into the center chamber.
- Once the buffer is dissolved, make sure to raise the two barrier combs (at the same time) so as to mix the total Dalt tank contents with the dissolved center chamber. Lower the combs; the tank is ready to run.

- Fill the gel casting box with cassettes interspersed with a total of 2 or 3 appropriately-sized sheets of "bubble-pack" material and a sheet of thin polycarbonate between each cassette.
- Prepare gel labels (section 17) and drop them into the cassettes on the side opposite the gradient inlet port.
- Place the Equalizer on one side on the top of the casting box, assigning one needle into each cassette.
- Insert the end of the gradient feed tube into the grommet in the floor of the side chamber of the casting box. **(If using Hoefer's ID 350 gradient maker, the gradient maker feed tube will be connected to a peristaltic pump, which in turn is connected to the casting box.)**
- Be sure that the gradient-maker lines are clamped off.
- Make up the gel acrylamide solutions from the stock mixes.
- Add the appropriate volumes of 10% ammonium persulfate and 10% TEMED and mix.
- Pour all the Light solution into the right side of the gradient maker (the side whose chamber is wider at the top).

• Carefully open the clamp on the Light chamber exit tube (#1), and then very slowly open the Heavy chamber exit tube clamp (#2) so as to allow Light solution to fill the tube coming from the Light chamber all the way to the "Y" connector and back to the point at which the Heavy tube enters the Heavy chamber.

• Close both clamps again. All three clamps are now closed.

• Add the Heavy solution to the Heavy (left) chamber until the liquid level reaches a point about 2 cm below the level of Light solution in the adjacent chamber.

• Fill the side chamber of the casting box with a dense displacing solution (50% glycerol or 35% sucrose).

• Carefully open the clamp on the Light exit tube (#1) and the clamp after the mixer (e.g., the feed tube clamp, #3). Light solution will begin to flow through the feed tube towards the casting box. (**Turn on the pump to bring the Light solution to the casting box.**)

• When the Light solution level in the gradient maker is about 1 cm above the level of the Heavy solution, open the Heavy exit tube clamp (#2).

• Cast the gels. (**With the ID 350, this will take approximately five minutes per box, assuming a pump setting of 280 ml/min for 7"x7" gels and 440 ml/min for 8"x10" gels.**)

• When the gradient maker is empty or the casting box is filled to within 1 cm from the top of the cassettes (whichever comes first), (**turn off the pump**) and close the feed tube clamp (#3). Remove the gradient feed tube and place its end in a waste receptacle for polymerizing acrylamide.

• When the acrylamide has drained (**or been pumped**) out, place the feed tube in the sink and pour about a liter of water into each chamber of the gradient maker. Open all clamps (**continue pumping**) to flush the system.

• Fill each of the syringes of the Equalizer with sec-butanol, buffer saturated. Let this overlay solution drop onto the top surface of the acrylamide. Check that all the syringes drop their contents.

• Continue flushing about 2 l more water through the gradient maker and tubing.

• Allow gels to polymerize for at least 1 hour.

• When polymerization is complete, remove the front of the casting box. The dense displacing solution will leak out at this point into the tray beneath the box.

• Carefully unload the cassettes from the box using a single-edged razor blade to separate the plates.

• Wash each cassette carefully with water to remove acrylamide adhered to the outer surfaces, catching the shreds of acrylamide in a fine mesh sink trap.

• Place the plates in alphabetical order in dishracks standing in plastic containers holding 1/4 in. of water.

• Place the Dalt gel in position with the rubber hinge strip to the side and the label readable from the front.

• With the aid of water pressure provided by a small syringe, extrude the Iso gel directly onto one of the cassette top edges (extending it along the edge with the acid end to the left). To make a suitable syringe gadget, cut the large end off a micropipette tip such that it will fit tightly on the Luer nipple of a 1 ml disposable plastic syringe. The syringe is filled with water, the tip inserted into the top end of a gel tube and the gel extruded by means of water pressure generated by pressing downwards on the syringe plunger. Pressure should be the minimum required

• Roll the rod gel over the edge of the cassette onto the Dalt gel with a squeeze bottle containing Iso equilibration buffer. Use this buffer to help lubricate the Iso. Smooth the gel into position with a spatula, eliminating any air bubbles underneath (between Iso and Dalt gels).

• Place a Wedgie into the loading cassette slot (don't push too hard but enough to hold the Iso against the Dalt gel). Alternatively, seal with agarose, allowing 2-5 minutes for the agarose to set up before going to the next step.

• Insert the cassette carefully into the Dalt tank, with the Iso gel vertical along the left side and the rubber cassette hinge along the bottom.

• Place a "buffer level raiser" (such as a gallon plastic bottle filled with water) into the tank.

• Close the lid on the tank. Attach the electrodes to make proper electrical contact with the power supply.

• Turn on the power supply and set a low voltage (≤50 volts) for running the gels.

• As each Dalt gel is ready to load, turn off the power supply, open the tank and insert the cassette. Then close the tank and restart the power supply while you prepare the next cassette.

• Remove the "buffer level raiser" once about half (10) of the gels have been loaded.

• After loading the last cassette, increase the voltage in the power supply to the desired run value and run the gels until the blue tracking dye reaches the right side of the gel holder (the "bottom" of the gel).

• When you are ready to remove the cassettes, turn off the power supply first.

• Remove the cassettes carefully, one at a time, using the fingers of both hands, and place them, 10 per rack, into dishracks standing in 1/4 in. of water.

• Carefully remove the Wedgies from the cassettes, rinse with abundant water and place them to dry.

• Open each cassette carefully, making sure that the gel comes with one of the plates and is not sticking partly to both (to avoid tearing the gel).

• Use a razor blade to separate the gel from the spacers (if it lies on the plate with spacers attached) and run it along the gel bottom (between gel and plate) in either case.

• Peel the gel from the glass carefully, lifting it by the bottom (high %T end) and place it in a box of staining solution.

6.13 Figures: Dalt Gel Casting

The following pages illustrate the process of making gradient and non-gradient Dalt slab gels.

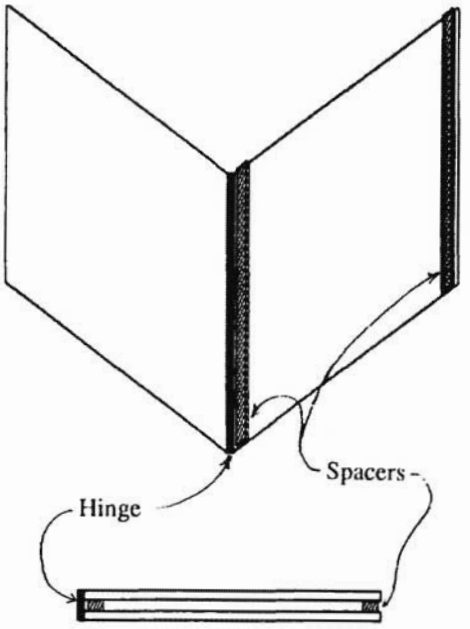

Figure 6.13.1: Dalt gel slab gel cassette, showing hinge and attached spacers.

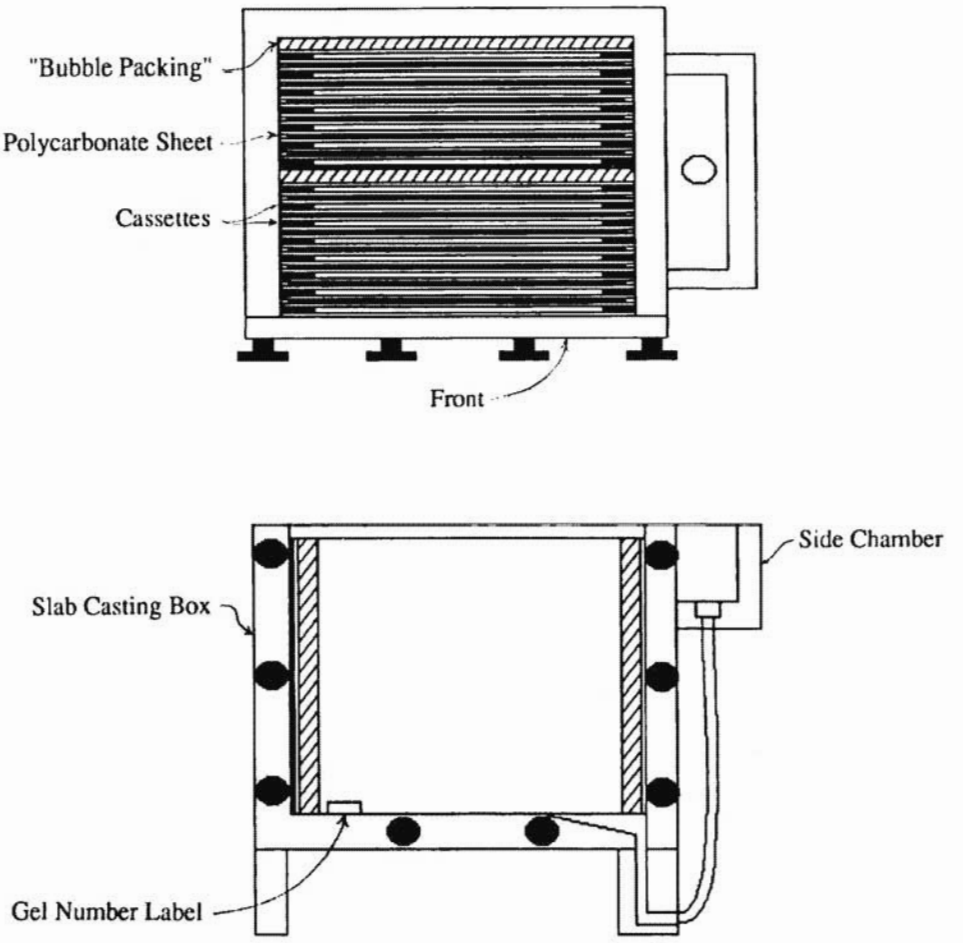

Figure 6.13.2: Top and front views of a Dalt slab gel casting box loaded with gel cassettes.

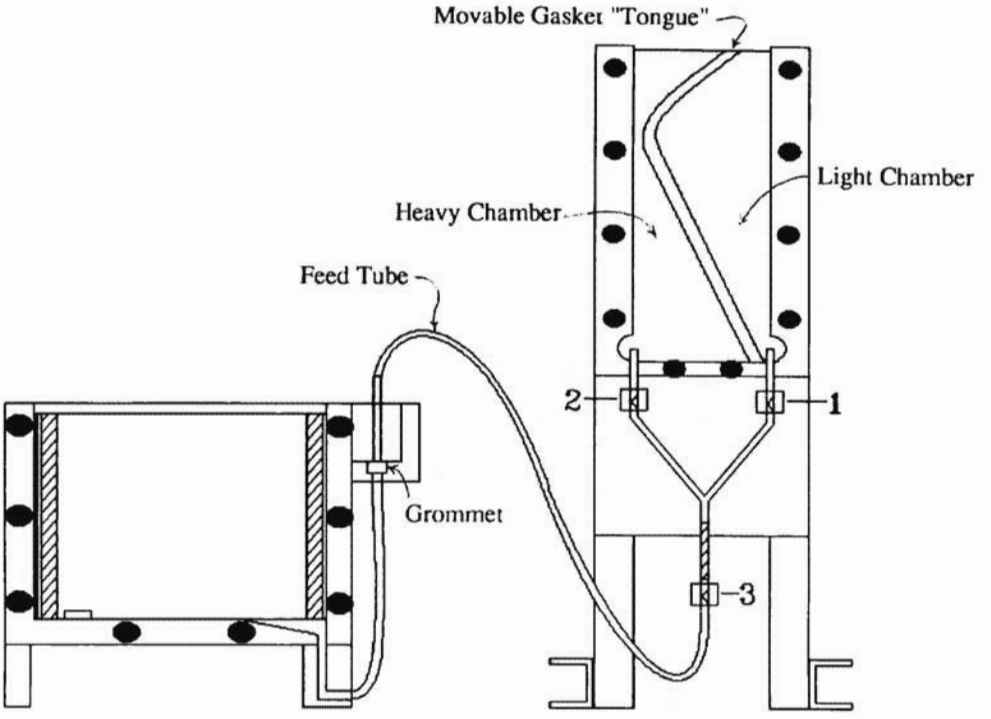

Figure 6.13.3: Setup of Dalt casting box and gradient maker. Gradient maker valves are numbered 1-3.

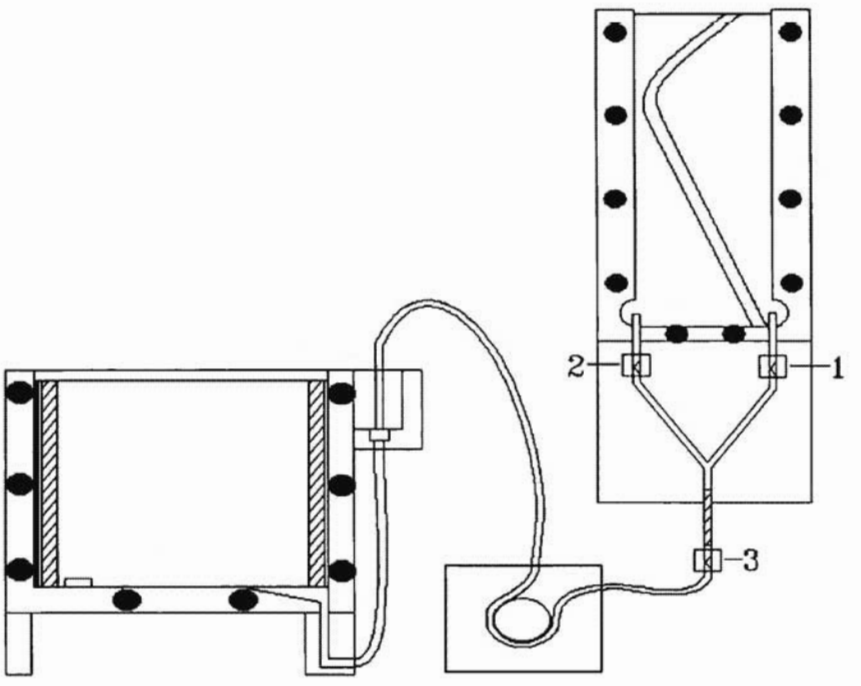

Figure 6.13.4: Setup of Dalt casting box and Hoefer's ID 350 pump-assisted gradient maker. Gradient maker valves are numbered 1-3.

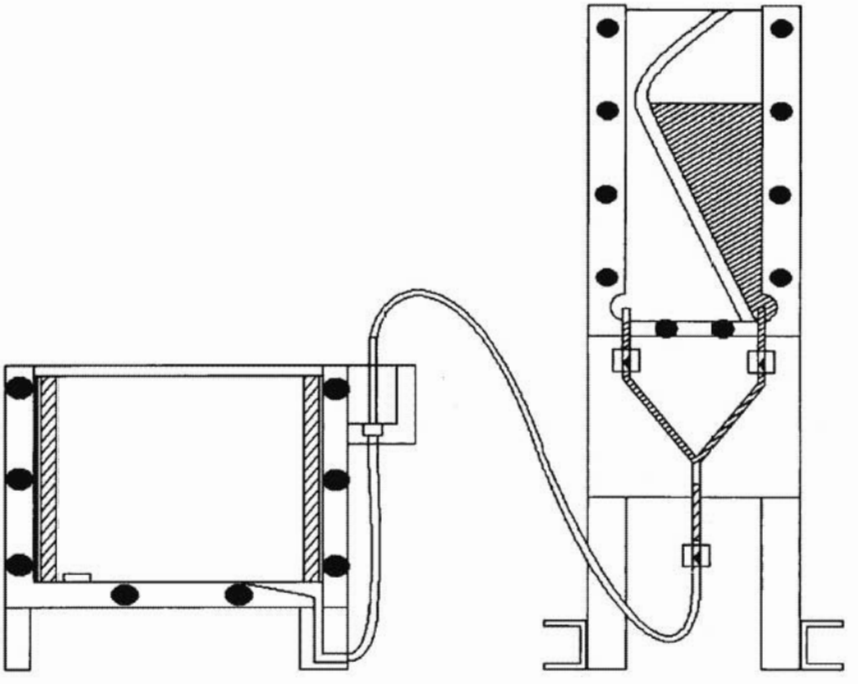

Figure 6.13.5: Dalt casting box and gradient maker with Light chamber filled.

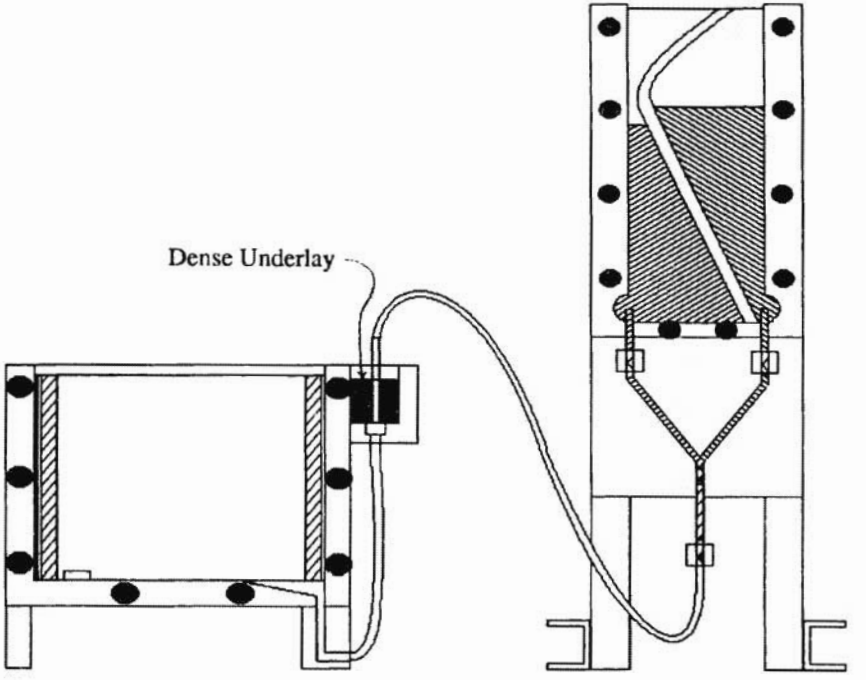

Figure 6.13.6: Dalt casting box (side chamber loaded with dense underlay) and gradient maker with both chambers filled, ready to pour gradient.

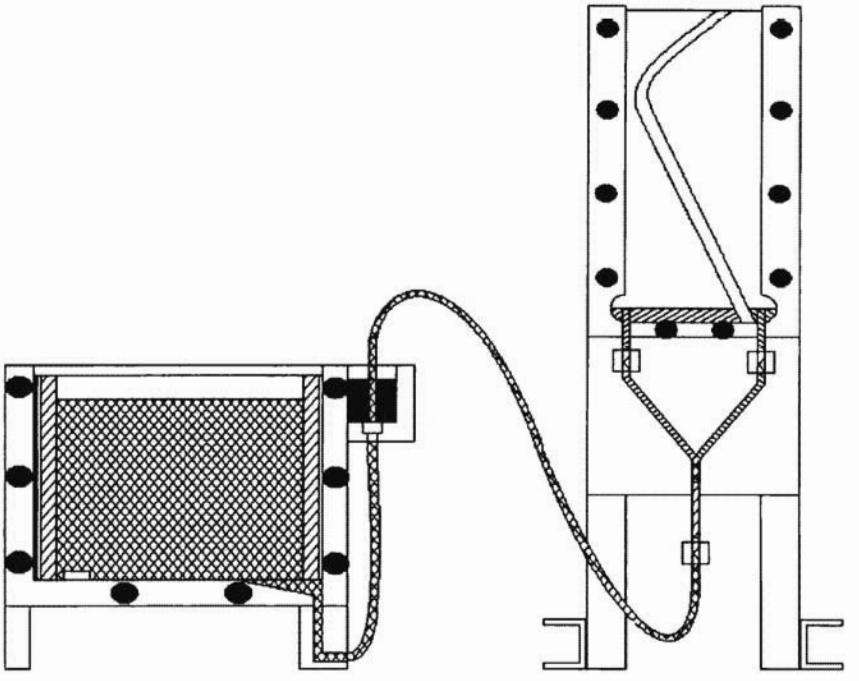

Figure 6.13.7: Dalt casting chamber after gradient is formed.

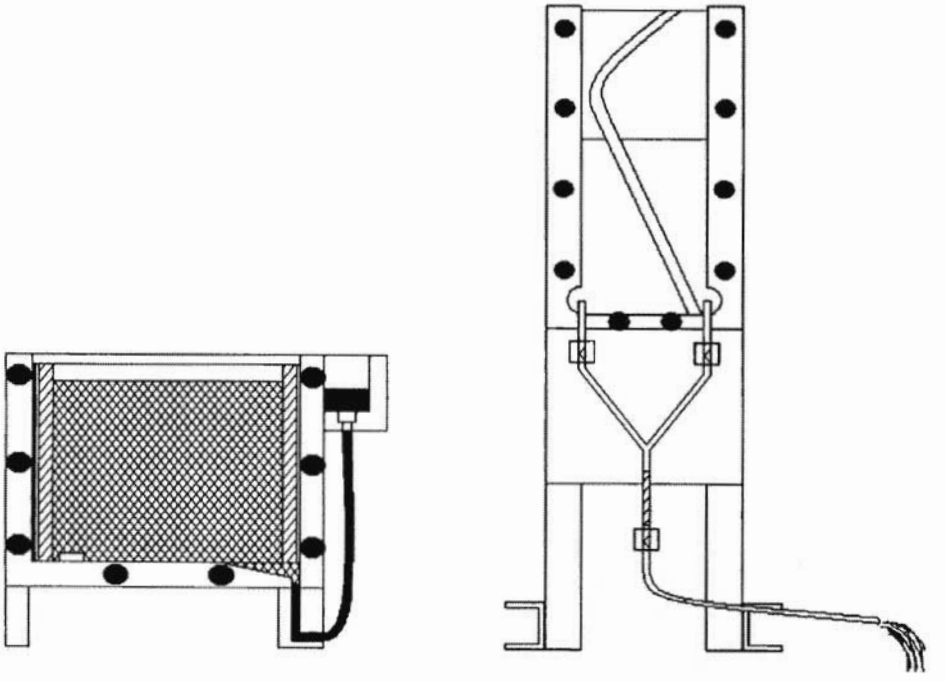

Figure 6.13.8: Dalt casting chamber with feed tube removed (underlay inflow started) and gradient maker rinsing to a waste receptacle.

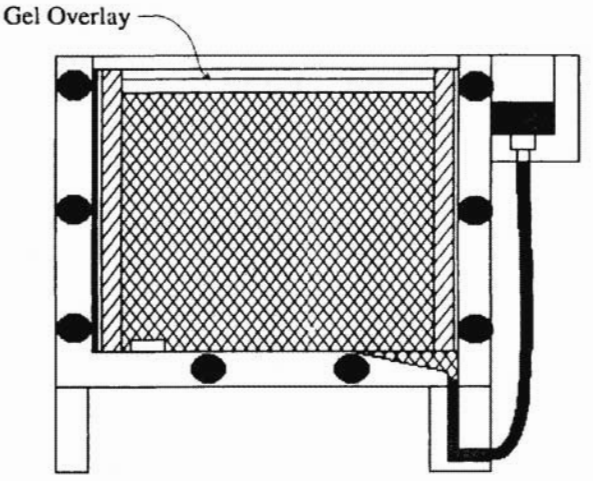

Figure 6.13.9: Dalt casting box with gel overlay applied.

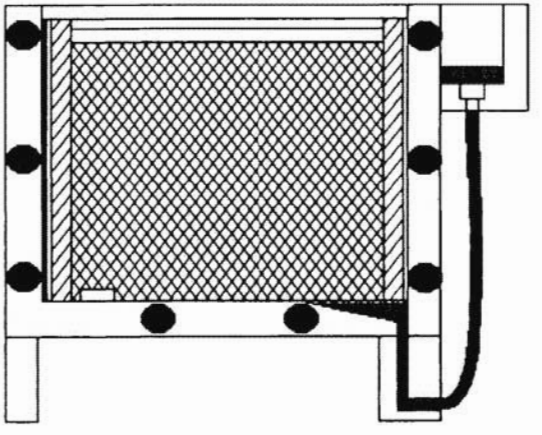

Figure 6.13.10: Dalt gel casting box after gel polymerization: Gel shrinkage draws underlay further into box.

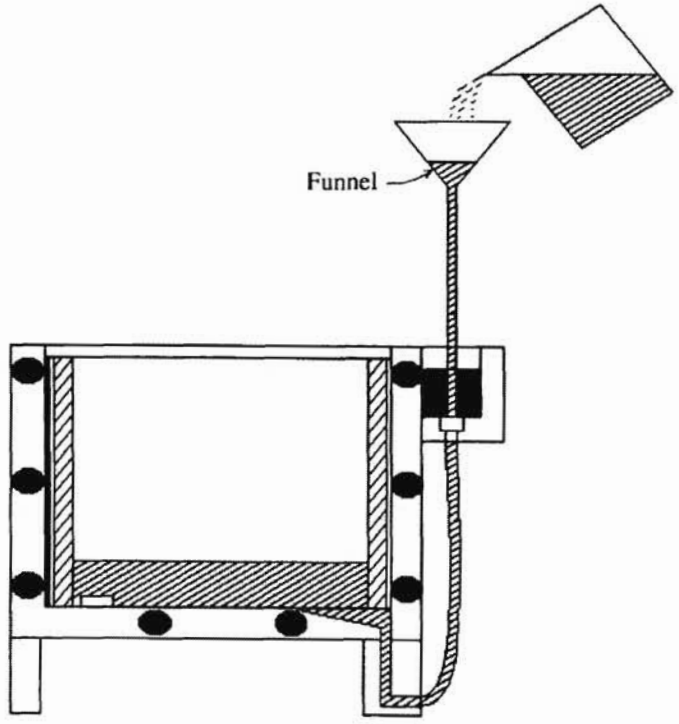

Figure 6.13.11: Casting non-gradient Dalt slab gels. Gradient maker is replaced by simple funnel.

6.14 Figures: Dalt Gel Loading, Running and Handling

The following figures show how Iso gels are loaded onto Dalt slabs, how these are run in the Dalt tank, and how the slabs are recovered for further processing.

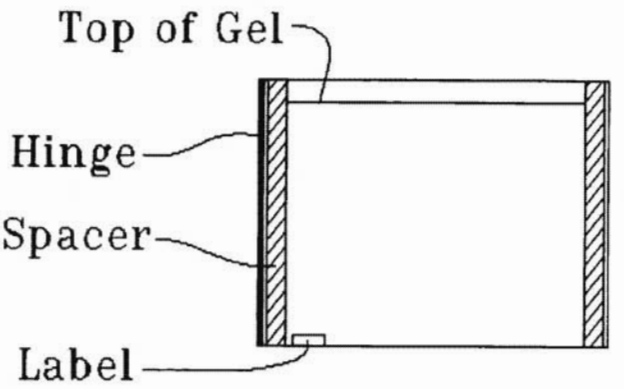

Figure 6.14.1: Dalt cassette containing a slab gel.

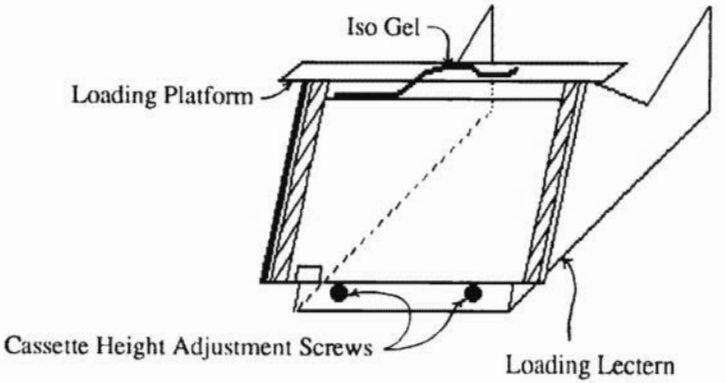

Figure 6.14.2: Dalt slab gel resting on loading lectern with Iso gel partly loaded on slab.

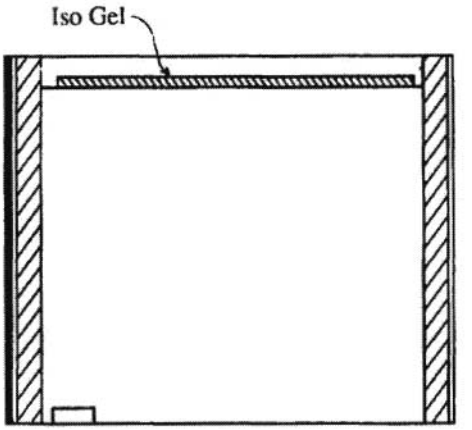

Figure 6.14.3: Dalt slab gel with Iso gel loaded.

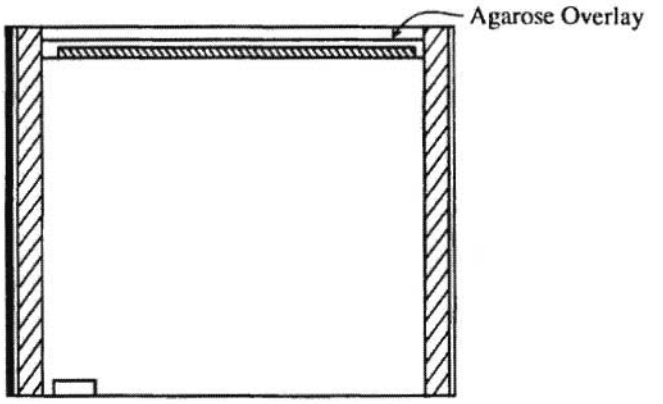

Figure 6.14.4: Dalt gel with agarose overlay holding Iso in place, ready for second dimension SDS electrophoresis.

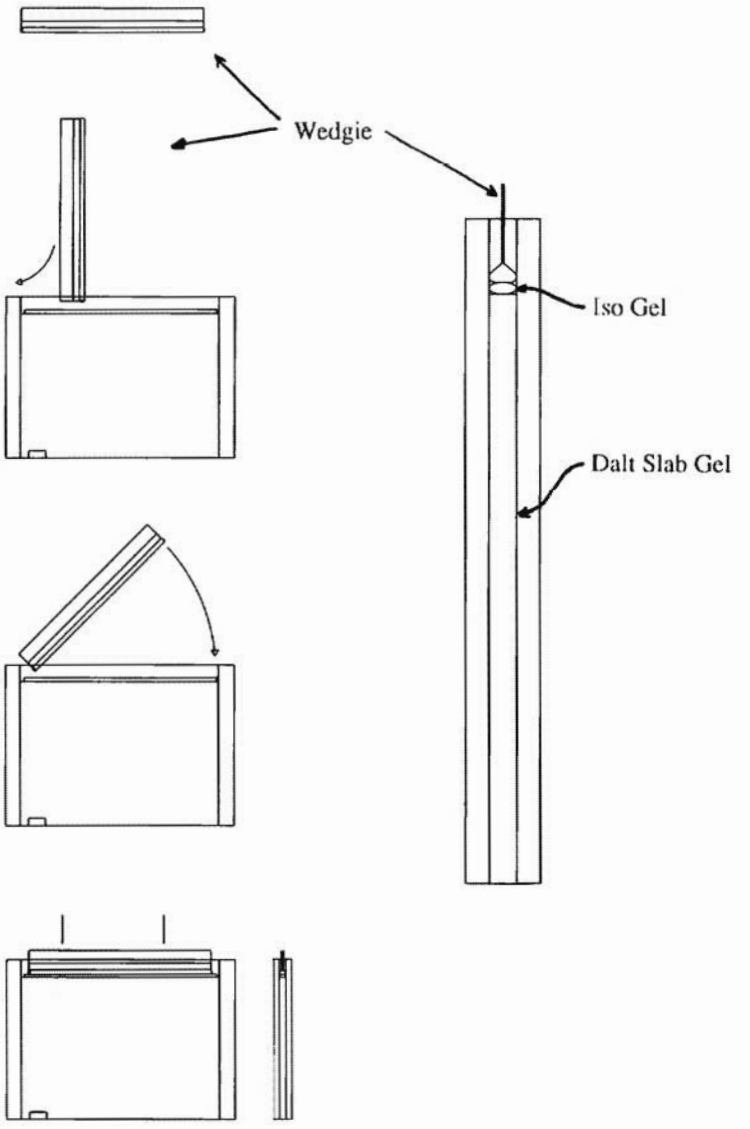

Figure 6.14.5: Insertion and correct positioning of Wedgie holding Iso rod gel smoothly against Dalt gel

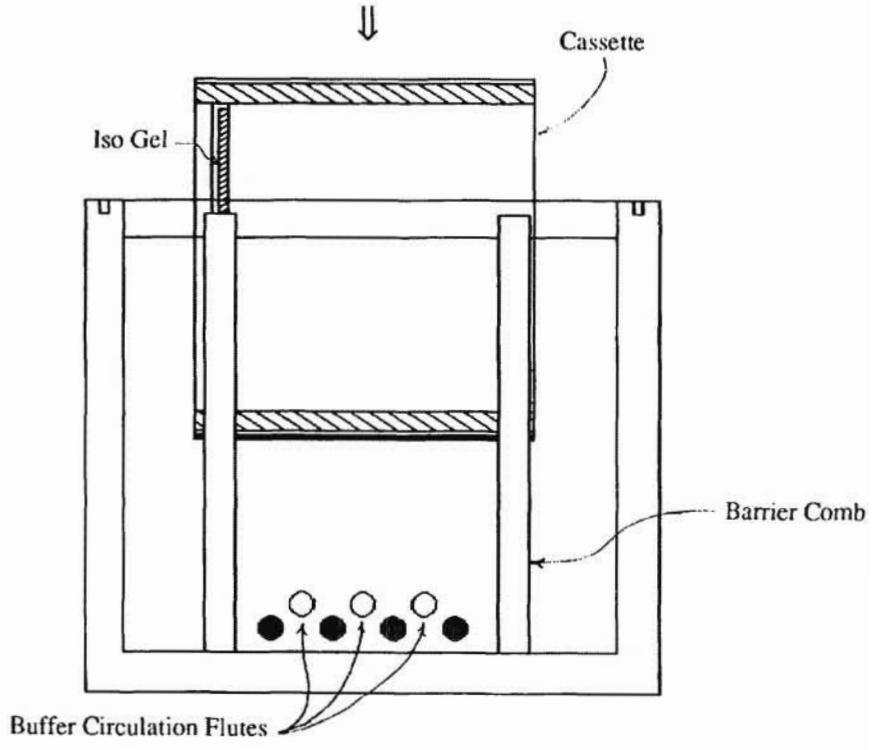

Figure 6.14.6: Insertion of loaded cassette into Dalt tank.
FRONT VIEW

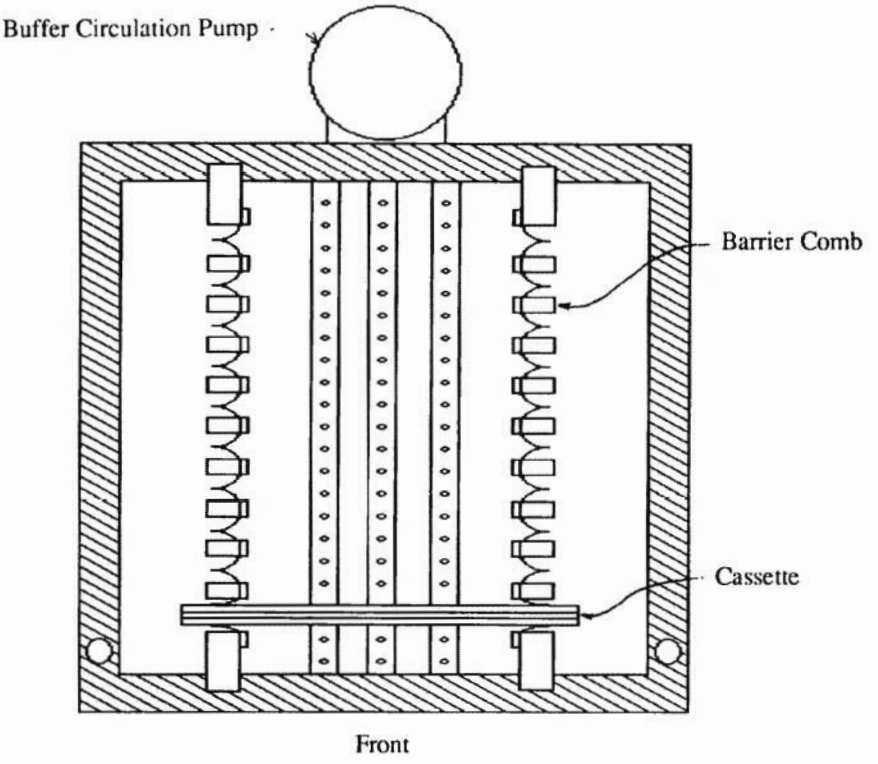

Figure 6.14.7: Insertion of loaded cassette into a 10-place Dalt tank.
TOP VIEW

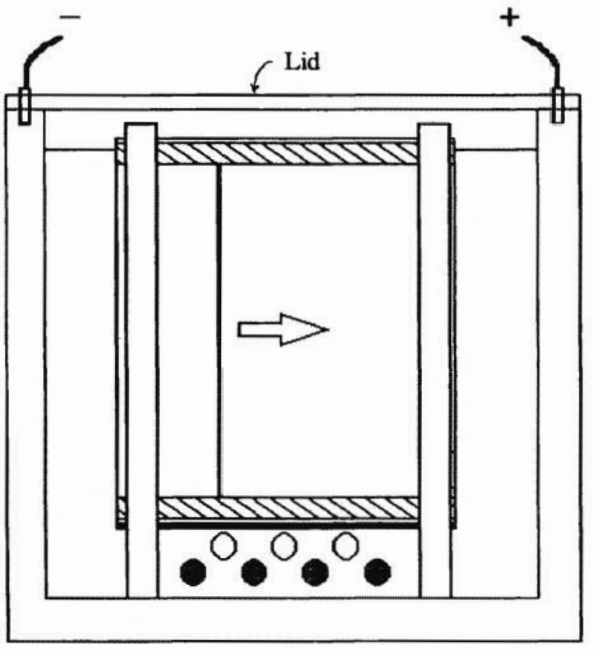

Figure 6.14.8: Electrophoresis in the Dalt tank, showing tracking dye progressing from left to right.

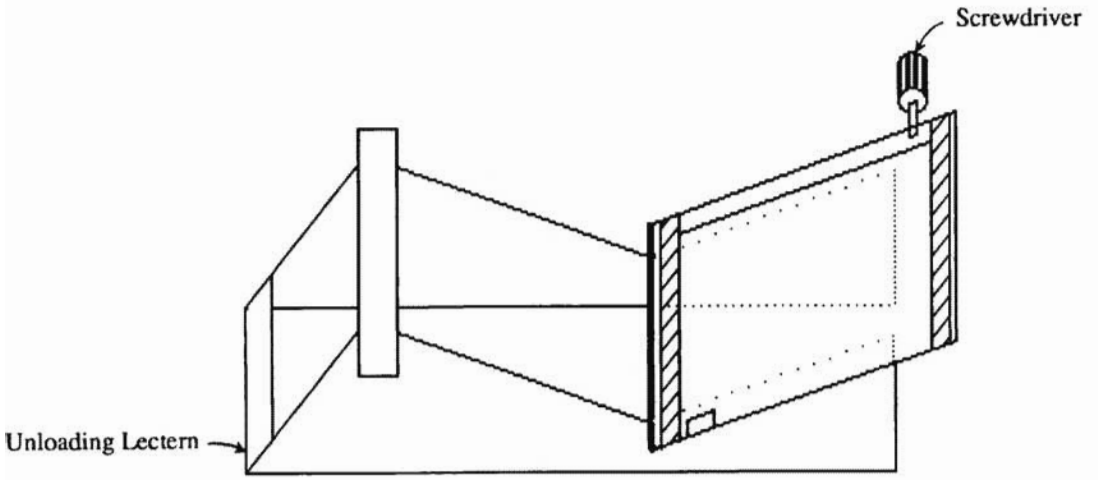

Figure 6.14.9: Opening Dalt cassette after run, using unloading lectern and a short screwdriver.

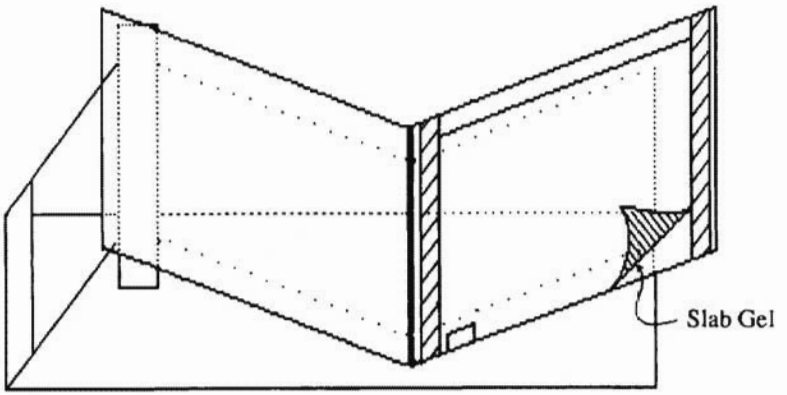

Figure 6.14.10: Removal of slab gel from opened Dalt cassette.

6.15 Dalt Slab Gel Recipes

The following tables give appropriate compositions for Light and Heavy components of gradients necessary to fill 22 cassettes (either 7"x7" [1400 ml total] or 8"x10" [2200 ml total]). Recipes are given for both volumetric and gravimetric procedures. Percentage compositions are also given in case other volumes are required. Modifications may be necessary for your laboratory.

Choose a Light mix from the left-hand page, determined by the percentage of total acrylamide (%T) desired at the top of the gel. Choose a Heavy mix from the facing (right-hand) page according to the %T desired at the bottom of the gel, subject to the requirement that $\%T_{Heavy} > \%T_{Light}$. An initial choice of 9% (Light) and 18% (Heavy) covers a wide range of applications.

Make the two solutions in beakers (1 or 2 l) so that you can easily pour them into the gradient maker chambers. Mix them well, but avoid making foam or bubbles.

If you plan to degas the solutions, do so before addition of the SDS, persulfate and TEMED.

Dalt Slab Gel Recipes Expressed as Percent

LIGHT

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
7%	22.73%	25.00%	50.03%	1.00%	0.00%	1.00%	0.244%
8%	25.97%	25.00%	46.81%	1.00%	0.00%	1.00%	0.214%
9%	29.22%	25.00%	43.59%	1.00%	0.00%	1.00%	0.190%
10%	32.47%	25.00%	40.36%	1.00%	0.00%	1.00%	0.171%
11%	35.71%	25.00%	37.13%	1.00%	0.00%	1.00%	0.155%
12%	38.96%	25.00%	33.90%	1.00%	0.00%	1.00%	0.143%
13%	42.21%	25.00%	30.66%	1.00%	0.00%	1.00%	0.132%
14%	45.45%	25.00%	27.42%	1.00%	0.00%	1.00%	0.122%
15%	48.70%	25.00%	24.18%	1.00%	0.00%	1.00%	0.114%
16%	51.95%	25.00%	20.95%	1.00%	0.00%	1.00%	0.107%
17%	55.19%	25.00%	17.70%	1.00%	0.00%	1.00%	0.101%
18%	58.44%	25.00%	14.46%	1.00%	0.00%	1.00%	0.095%
19%	61.69%	25.00%	11.22%	1.00%	0.00%	1.00%	0.090%

HEAVY

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
8%	25.97%	25.00%	39.45%	1.00%	8.00%	0.50%	0.071%
9%	29.22%	25.00%	36.22%	1.00%	8.00%	0.50%	0.063%
10%	32.47%	25.00%	32.98%	1.00%	8.00%	0.50%	0.057%
11%	35.71%	25.00%	29.73%	1.00%	8.00%	0.50%	0.052%
12%	38.96%	25.00%	26.49%	1.00%	8.00%	0.50%	0.047%
13%	42.21%	25.00%	23.25%	1.00%	8.00%	0.50%	0.044%
14%	45.45%	25.00%	20.00%	1.00%	8.00%	0.50%	0.041%
15%	48.70%	25.00%	16.76%	1.00%	8.00%	0.50%	0.038%
16%	51.95%	25.00%	13.52%	1.00%	8.00%	0.50%	0.036%
17%	55.19%	25.00%	10.27%	1.00%	8.00%	0.50%	0.034%
18%	58.44%	25.00%	7.03%	1.00%	8.00%	0.50%	0.032%
19%	61.69%	25.00%	3.78%	1.00%	8.00%	0.50%	0.030%
20%	64.94%	25.00%	0.54%	1.00%	8.00%	0.50%	0.029%

For 1100 ml each Heavy and Light (22 8"x10" gels):
BY VOLUME in ml

LIGHT

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
7%	250.00	275.00	550.31	11.00	0.00	11.00	2.69
8%	285.71	275.00	514.93	11.00	0.00	11.00	2.35
9%	321.43	275.00	479.48	11.00	0.00	11.00	2.09
10%	357.14	275.00	443.98	11.00	0.00	11.00	1.88
11%	392.86	275.00	408.43	11.00	0.00	11.00	1.71
12%	428.57	275.00	372.86	11.00	0.00	11.00	1.57
13%	464.29	275.00	337.27	11.00	0.00	11.00	1.45
14%	500.00	275.00	301.66	11.00	0.00	11.00	1.34
15%	535.71	275.00	266.03	11.00	0.00	11.00	1.25
16%	571.43	275.00	230.40	11.00	0.00	11.00	1.18
17%	607.14	275.00	194.75	11.00	0.00	11.00	1.11
18%	642.86	275.00	159.10	11.00	0.00	11.00	1.05
19%	678.57	275.00	123.44	11.00	0.00	11.00	0.99

HEAVY

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
8%	285.71	275.00	434.00	11.00	88.00	5.50	0.78
9%	321.43	275.00	398.37	11.00	88.00	5.50	0.70
10%	357.14	275.00	362.73	11.00	88.00	5.50	0.63
11%	392.86	275.00	327.07	11.00	88.00	5.50	0.57
12%	428.57	275.00	291.41	11.00	88.00	5.50	0.52
13%	464.29	275.00	255.73	11.00	88.00	5.50	0.48
14%	500.00	275.00	220.05	11.00	88.00	5.50	0.45
15%	535.71	275.00	184.37	11.00	88.00	5.50	0.42
16%	571.43	275.00	148.68	11.00	88.00	5.50	0.39
17%	607.14	275.00	112.99	11.00	88.00	5.50	0.37
18%	642.86	275.00	77.29	11.00	88.00	5.50	0.35
19%	678.57	275.00	41.60	11.00	88.00	5.50	0.33
20%	714.29	275.00	5.90	11.00	88.00	5.50	0.31

For approximately 1150 g each Heavy and Light:
BY WEIGHT in g (except Persulfate and TEMED: ml)

LIGHT

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
7%	257.50	289.58	550.31	11.12	0.00	11.00	2.69
8%	294.29	289.58	514.93	11.12	0.00	11.00	2.35
9%	331.07	289.58	479.48	11.12	0.00	11.00	2.09
10%	367.86	289.58	443.98	11.12	0.00	11.00	1.88
11%	404.64	289.58	408.43	11.12	0.00	11.00	1.71
12%	441.43	289.58	372.86	11.12	0.00	11.00	1.57
13%	478.21	289.58	337.27	11.12	0.00	11.00	1.45
14%	515.00	289.58	301.66	11.12	0.00	11.00	1.34
15%	551.79	289.58	266.03	11.12	0.00	11.00	1.25
16%	588.57	289.58	230.40	11.12	0.00	11.00	1.18
17%	625.36	289.58	194.75	11.12	0.00	11.00	1.11
18%	662.14	289.58	159.10	11.12	0.00	11.00	1.05
19%	698.93	289.58	123.44	11.12	0.00	11.00	0.99

HEAVY

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
8%	294.29	289.58	434.00	11.12	110.11	5.50	0.78
9%	331.07	289.58	398.37	11.12	110.11	5.50	0.70
10%	367.86	289.58	362.73	11.12	110.11	5.50	0.63
11%	404.64	289.58	327.07	11.12	110.11	5.50	0.57
12%	441.43	289.58	291.41	11.12	110.11	5.50	0.52
13%	478.21	289.58	255.73	11.12	110.11	5.50	0.48
14%	515.00	289.58	220.05	11.12	110.11	5.50	0.45
15%	551.79	289.58	184.37	11.12	110.11	5.50	0.42
16%	588.57	289.58	148.68	11.12	110.11	5.50	0.39
17%	625.36	289.58	112.99	11.12	110.11	5.50	0.37
18%	662.14	289.58	77.29	11.12	110.11	5.50	0.35
19%	698.93	289.58	41.60	11.12	110.11	5.50	0.33
20%	735.71	289.58	5.90	11.12	110.11	5.50	0.31

For 700 ml each Heavy and Light (22 7"x7" gels):
BY VOLUME in ml

LIGHT

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
7%	159.09	175.00	350.20	7.00	0.00	7.00	1.71
8%	181.82	175.00	327.69	7.00	0.00	7.00	1.50
9%	204.55	175.00	305.12	7.00	0.00	7.00	1.33
10%	227.27	175.00	282.53	7.00	0.00	7.00	1.20
11%	250.00	175.00	259.91	7.00	0.00	7.00	1.09
12%	272.73	175.00	237.28	7.00	0.00	7.00	1.00
13%	295.45	175.00	214.62	7.00	0.00	7.00	0.92
14%	318.18	175.00	191.96	7.00	0.00	7.00	0.85
15%	340.91	175.00	169.29	7.00	0.00	7.00	0.80
16%	363.64	175.00	146.62	7.00	0.00	7.00	0.75
17%	386.36	175.00	123.93	7.00	0.00	7.00	0.70
18%	409.09	175.00	101.24	7.00	0.00	7.00	0.66
19%	431.82	175.00	78.55	7.00	0.00	7.00	0.63

HEAVY

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
8%	181.82	175.00	276.18	7.00	56.00	3.50	0.50
9%	204.55	175.00	253.51	7.00	56.00	3.50	0.44
10%	227.27	175.00	230.83	7.00	56.00	3.50	0.40
11%	250.00	175.00	208.14	7.00	56.00	3.50	0.36
12%	272.73	175.00	185.44	7.00	56.00	3.50	0.33
13%	295.45	175.00	162.74	7.00	56.00	3.50	0.31
14%	318.18	175.00	140.03	7.00	56.00	3.50	0.28
15%	340.91	175.00	117.32	7.00	56.00	3.50	0.27
16%	363.64	175.00	94.61	7.00	56.00	3.50	0.25
17%	386.36	175.00	71.90	7.00	56.00	3.50	0.23
18%	409.09	175.00	49.19	7.00	56.00	3.50	0.22
19%	431.82	175.00	26.47	7.00	56.00	3.50	0.21
20%	454.55	175.00	3.76	7.00	56.00	3.50	0.20

For approximately 730 g each Heavy and Light:
BY WEIGHT in g (except Persulfate and TEMED: ml)

LIGHT

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
7%	163.86	184.28	350.20	7.08	0.00	7.00	1.71
8%	187.27	184.28	327.69	7.08	0.00	7.00	1.50
9%	210.68	184.28	305.12	7.08	0.00	7.00	1.33
10%	234.09	184.28	282.53	7.08	0.00	7.00	1.20
11%	257.50	184.28	259.91	7.08	0.00	7.00	1.09
12%	280.91	184.28	237.28	7.08	0.00	7.00	1.00
13%	304.32	184.28	214.62	7.08	0.00	7.00	0.92
14%	327.73	184.28	191.96	7.08	0.00	7.00	0.85
15%	351.14	184.28	169.29	7.08	0.00	7.00	0.80
16%	374.55	184.28	146.62	7.08	0.00	7.00	0.75
17%	397.95	184.28	123.93	7.08	0.00	7.00	0.70
18%	421.36	184.28	101.24	7.08	0.00	7.00	0.66
19%	444.77	184.28	78.55	7.08	0.00	7.00	0.63

HEAVY

%T	Acryl. 30.8%	Buffer "D"	Water	SDS 10%	Glycerol	Am Pers 10%	TEMED 10%
8%	187.27	184.28	276.18	7.08	70.07	3.50	0.50
9%	210.68	184.28	253.51	7.08	70.07	3.50	0.44
10%	234.09	184.28	230.83	7.08	70.07	3.50	0.40
11%	257.50	184.28	208.14	7.08	70.07	3.50	0.36
12%	280.91	184.28	185.44	7.08	70.07	3.50	0.33
13%	304.32	184.28	162.74	7.08	70.07	3.50	0.31
14%	327.73	184.28	140.03	7.08	70.07	3.50	0.28
15%	351.14	184.28	117.32	7.08	70.07	3.50	0.27
16%	374.55	184.28	94.61	7.08	70.07	3.50	0.25
17%	397.95	184.28	71.90	7.08	70.07	3.50	0.23
18%	421.36	184.28	49.19	7.08	70.07	3.50	0.22
19%	444.77	184.28	26.47	7.08	70.07	3.50	0.21
20%	468.18	184.28	3.76	7.08	70.07	3.50	0.20

6.16 Dalt Notes

Dalt Notes

7 The Angelique™ Computer-Controlled Gradient Maker

This section outlines the operation of the Angelique computer-controlled gradient maker. The purpose of this device is to allow the user to produce acrylamide gradient slab gels easily and reproducibly. Using an IBM-compatible personal computer, the system's software is implemented as a Lotus 1-2-3® spreadsheet that drives stepper motors through a serial interface. These motors in turn drive two sets of peristaltic pumps, one set for the LIGHT acrylamide mixture and the other for the HEAVY mixture. By varying the ratio between these two mixtures, a wide variety of gradients can be produced. In addition, the software includes options for calibrating the pumps, for storing and retrieving various gradients, and for keeping a running log of the gradients made (with the stock solution batches used).

Angelique version 8, described here, is specifically tailored to allow use of "pre-made" acrylamide stock solutions (e.g., 30.8%T) available from several sources.

While basic familiarity with PC operation is assumed, the user need not be familiar with operation of the Lotus 1-2-3 spreadsheet program itself. However, since your Angelique system includes a fully-functional copy of 1-2-3, you are encouraged to make use of its capabilities for other laboratory tasks (**23**), provided the basic setup on which Angelique depends is not changed.

7.1 Selecting Options

Select an option from a menu across the top of the screen by one of two methods:

- move the bright cursor block over to the desired choice and hit [Return], or
- type the first letter of the desired menu choice (you'll notice all choices on the same menu start with different first letters).

Placing the cursor on an option automatically brings up a one line explanation of its use on the second line of the display.

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23) LSB cannot, of course, provide any support for such additional uses.

7.2 Entering Requested Data

When a requested option asks for data, enter the value, name or comment needed and finish with a [Return]. When numbers are required, enter these without commas (as you might in 21,000) but with decimal points (if necessary).

7.3 Installation

Angelique comes in four boxes; two for the PC, one for the pump module and one for the pump controller. Unpack these carefully.

Place the PC chassis on top of the short grey 19" rack containing the pump controller, with the PC monitor on top of the PC and the PC keyboard on the black lucite shelf (which comes packed with the pump module and is to be mounted on the front of the rack).

Connect the PC keyboard, the monitor power and the monitor signal to the respective plugs on the back of the PC. Connect the PC's power cord to the usual 110V wall socket (**24**).

Remove the cardboard protector from the PC's floppy drive.

Connect the pump controller power cord (with the line switch on it) to a 110V wall socket (with the line switch initially in the off position) (**25**). Connect the 9-pin cable from the controller rack to the COM1 port on the back of the PC. Run the long blue cable from the controller rack to the site of the pumping module (on the gel casting bench).

Place the pump module on the casting bench, and connect the long blue cable to the mating connector on the cable from the pump module. It is a good idea to tighten the little screws that hold the two halves of this connector together. Remove the tape and packing from the tubes.

---

24) In areas with 220V, 50Hz power, you can operate the system from a step-down transformer supplying 110V. The PC can be operated directly from 220V, 50Hz by taking the following two steps: switch the small switch on the back of the PC (just above the power cord socket) from 115 to 220, and then convert the monitor by removing the plastic case (6 screws) and clipping jumper W13. Be sure to carry out such operations only with all power off.

25) The pump controller system requires 110V; in areas where 220V is conventional, a step-down transformer must be used. If this is not possible, consult LSB.

The seven intake tubes are labelled as to the solution reservoirs they go into. The four TEMED/Persulfate reservoirs should hold about 500 ml each, while the Buffer/Acrylamide reservoirs should hold more than 1 liter (preferably 2 or 3).

The gradient delivery tube emerging from the top of the lucite mixing manifold goes into the grommet in the gel casting box.

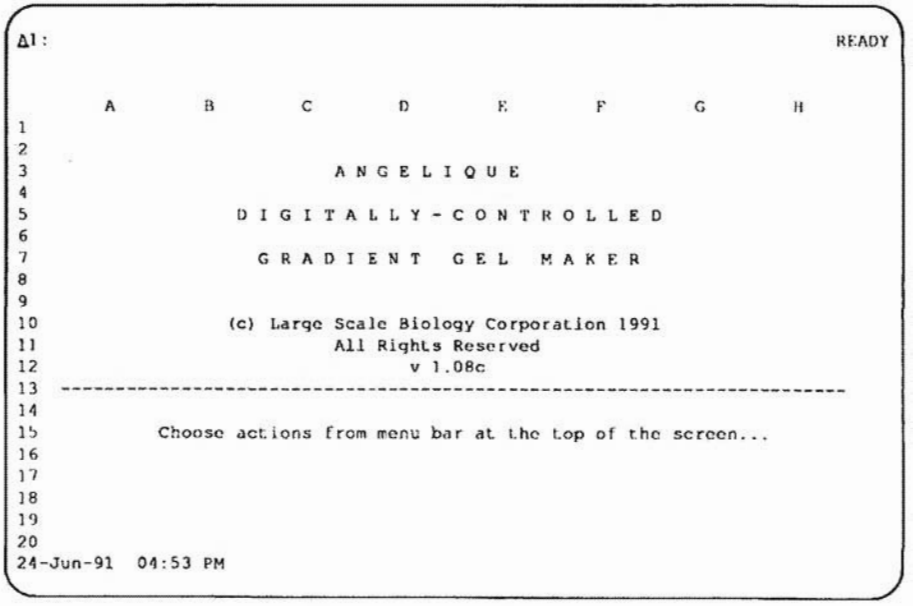
7.4 Starting Angelique

Start the PC by turning on the computer power switch and the monitor (if this is plugged in separately). The computer will then execute its internal diagnostics and finally produce the MS-DOS prompt: "C:>".

Then type what appears below in **bold** at the appropriate times:

C:> **CD \123** Return	Go to the Lotus directory.
C:> **123** Return	Loads Lotus 1-2-3 and then the Measure® add-in software.
q Return	Complete loading of the Measure add-in software.
/FRANGEL Return when the READY signal appears at the upper right of the screen.	Once inside Lotus 1-2-3 this command loads the ANGEL spreadsheet that controls the gradient maker

The following screen appears as Angelique starts operation:


A1:
READY
A B C D E F G H
1
2
3 ANGELIQUE
4
5 DIGITALLY-CONTROLLED
6
7 GRADIENT GEL MAKER
8
9
10 (c) Large Scale Biology Corporation 1991
11 All Rights Reserved
12 v 1.08c
13
14
15 Choose actions from menu bar at the top of the screen...
16
17
18
19
20
24-Jun-91 04:53 PM

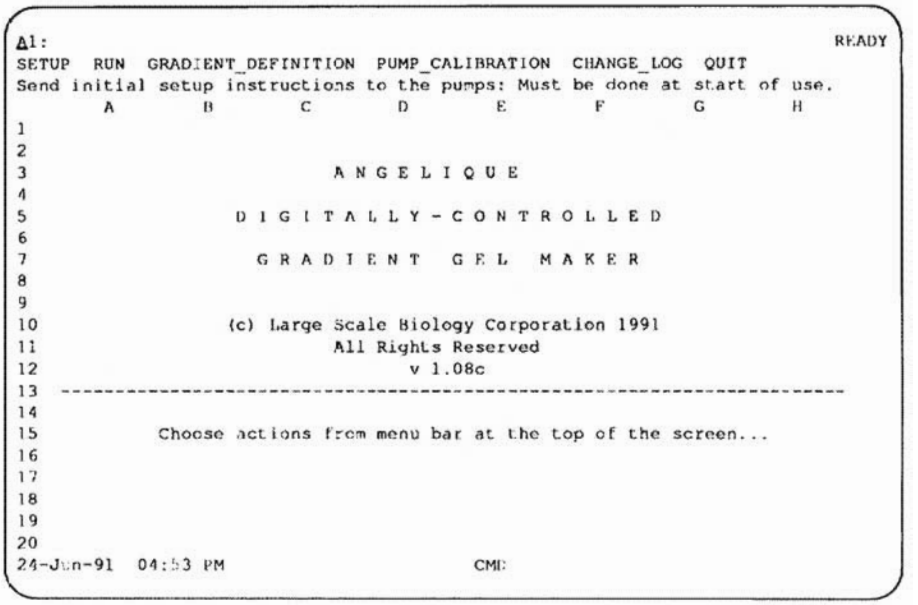
7.5 Routine Operation

The routine sequence of operations is roughly as follows:

- Turn on Angelique (both computer and pump controller).
- Start the software as described in 7.4.
- Select the SET_UP option to set up the pump controller for correct operation (this step is required every time the power to the pump controller unit is first turned on).
- Choose the GRADIENT_DEFINITION option and either create, recall or modify the gradient information to get the desired design. Exit using the QUIT option: this will load the pump controller with the gradient information.
- Choose RUN from the Top Menu.
- Place the five intake tubes in the correct solution reservoirs and place the delivery tube in a waste container (to receive polymerizable acrylamide).
- PRIME the pumps, making sure that all air bubbles are purged from the pumps and the delivery tube.
- FLUSH the delivery tube in preparation for the gradient (the delivery tube is still in the waste reservoir).
- Place the delivery tube in the casting box grommet, load the side chamber with dense solution, and use GRADIENT to pump the gradient into the box.
- Repeat the FLUSH, GRADIENT, FLUSH, GRADIENT, FLUSH..... cycle as required for the number of casting boxes to be filled.
- Finally, place the intake tubes in a reservoir of water and use PRIME to rinse all the acrylamide and other solutions out of the system.

7.6 Main Menu

When Angel is loaded, a menu (called the Top Menu) is displayed along the top of the screen:



When starting the system, always choose SETUP first, as this performs the necessary initial tasks involved in starting the pump controllers (which must be turned on to receive the commands). QUIT stops Angelique and returns to the DOS prompt.

The following sections (7.7 - 7.11) describe the submenus or commands accessible from the Main Menu.

7.7 SETUP

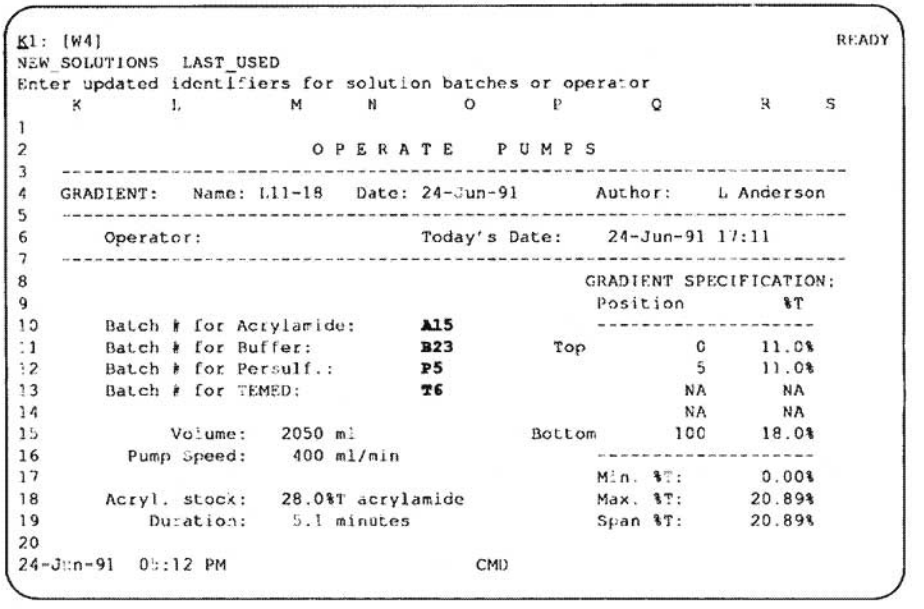
SETUP sends a series of commands to the pump controller to set up parameters and check that the controller is on. This must be carried out before any pump actions are attempted. Do it first thing on startup, being sure that the pump controller power switch is on (the line cord switch).

When the controller receives the SETUP commands, you will notice that a small red LED (visible through a small hole in the right side black "Compumotor" cover-plate) will flash several times and then stay off. If the red light remains on, then the controller has detected a problem and will not proceed (see section 7.12).

If the pumps are not on, or if the pumps do not acknowledge the commands sent to them, then a message will appear at the bottom of the screen alerting the user to a potential problem. On encountering such a problem, the best solution is to turn off the pump controller power (with the line switch), exit the program, and restart from the beginning.

7.8 RUN

RUN allows access to a submenu of pumping commands. First, however, the user is required to enter the batch numbers of solutions to be used in the casting run.



The user can select to use the batch identifiers shown on the screen **as bold** values by selecting LAST_USED. This signifies that you will be using all the same solution batches as you used in the last gel casting run. If you are using a different batch of one or more of the seven stock solutions, you will want to enter identifying batch numbers for these solutions, and should select NEW_SOLUTIONS. This choice will result in the user being prompted for the new identifiers one after another. These will then be placed on the screen in bold.

These batch labels are important since they are written out into a "log" file along with information on the gradient used, date, etc., for each gradient produced (see section 7.11). This log can be useful in quality control.

Once the solutions are identified, the main RUN menu appears:

```
K1: [W4]                                                          READY
GRADIENT  FLUSH  PRIME/PURGE  IDLE  DENSE  STOP  BATCHES  QUIT
Pump a gradient as specified by the current parameters
     K         L          M      N        O       P        Q        R     S
1
2                          O P E R A T E    P U M P S
3   ------------------------------------------------------------------------
4   GRADIENT:   Name: L11-18   Date: 24-Jun-91       Author:    L Anderson
5   ------------------------------------------------------------------------
6       Operator:      reb            Today's Date:    24-Jun-91 17:12
7   ------------------------------------------------------------------------
8                                                    GRADIENT SPECIFICATION:
9                                                     Position        %T
10      Batch # for Acrylamide:      6/20/91          --------------------
11      Batch # for Buffer:          6/20/91     Top          0      11.0%
12      Batch # for Persulf.:        5/14/91                  5      11.0%
13      Batch # for TEMED:           6/20/91                 NA       NA
14                                                           NA       NA
15            Volume:   2050 ml                 Bottom      100      18.0%
16        Pump Speed:    400 ml/min                        --------------------
17                                                    Min. %T:        0.00%
18      Acryl. stock:   28.0%T acrylamide             Max. %T:       20.89%
19          Duration:    5.1 minutes                  Span %T:       20.89%
20
24-Jun-91  05:12 PM                        CMD
```

The screen shows:

- Information about the gradient to be pumped:

  Name
  Date entered
  Author

  The gradient specification, the minimum and maximum %T that could be obtained with these solutions.

  The volume to be pumped, the pump speed, the concentrations specified for the light and heavy acrylamide stock solutions, and the expected duration of the pumping operation.

- Information about the operator casting these gels and the date of casting.

In running the pumps, remember:

IF A MOTION IS IN PROGRESS, USE STOP TO HALT IT BEFORE CHOOSING THE NEXT MOTION.

DON'T CHOOSE A NEW MOTION BEFORE THE MENU HAS REAPPEARED AFTER THE LAST MOTION.

IF THE SMALL RED LIGHT COMES ON (ON THE CONTROLLER FRONT PLATE) AND YOU CAN'T GET A NEW MOTION STARTED, HIT STOP TO CLEAR THE SYSTEM.

The menu choices shown allow the following operations of the pumps, all of which begin after a short pause once the selection is made:

7.8.1 GRADIENT

Pumps a gradient as specified by the last set of parameters set in the GRADIENT_DEFINITION option of the Top Menu. Before beginning, the program asks for a batch number for the gels being cast. This number is written into the log file to enable tracking of gels by batch number.

The feed to the casting box will consist of an initial small segment of constant composition material generated by the previous FLUSH cycle (the contents of the gradient feed tube; see 7.8.2) followed by the gradient as specified (from beginning to end, light to heavy). The GRADIENT option follows the gradient with a volume of bottom composition material equal in volume to the delivery tube, so that the gradient is completely delivered into the casting box.

7.8.2 FLUSH

Flushes out the pumps and delivery tube in preparation for pumping a gradient. This step is necessary between gradients because the mixing chamber and delivery tube are filled (at the end of the gradient) with a composition appropriate to the bottom of the gel, whereas the next gradient run must deliver, from the first moment, a composition appropriate for the top of the gel. Thus an intermediate step is required, namely flushing out the bottom mixture from the tubes and replacing it with the top mixture.

Using this approach, the first material pumped into the gel box will be a segment of gel mix of volume equal to the delivery tube and composition equal to that specified for the top of the gradient. This is typically 65 ml, or 3% of the gradient. After this the gradient as specified will begin.

Always make sure that the output stream from the gradient delivery tube is going into a waste vessel (where it can polymerize without harm before disposal) when FLUSHing the system.

7.8.3 PRIME/PURGE

Primes the pumps. When the system is being started up and the intake tubes are placed in the solution reservoirs, this action is used to draw solutions into the pumping system and purge air (or wash water) from the tubing. The number of cycles to be pumped can be specified (1-10); one cycle is roughly sufficient to draw liquid from the input tubes to the end of the output feed tube. Three cycles are advisable as a final washout.

This option is also useful for rinsing out the system with water (or finally with air) after use.

Always make sure that the output stream from the gradient delivery tube is going into a waste vessel (where it can polymerize without harm before disposal) when PRIMEing the system.

7.8.4 IDLE

Drives the pumps slowly, thereby preventing polymerization of acrylamide in the system. This option allows the system to be left loaded with polymerizable solutions for brief periods. For longer periods between runs, it is recommended that the acrylamide, etc., be flushed out of the entire system with water.

7.8.5 DENSE

Pumps a continuous stream of the final (most dense, i.e., bottom) gel composition. This option is used if, for some reason, the gradient volume was not large enough to fill the gel cassettes to the desired height. By pumping the DENSE composition immediately after the end of the gradient, one can continue to raise the level in the cassettes.

This option must be ended by choosing STOP. Otherwise it will continue indefinitely...

7.8.6 STOP

Stops the pumps. This option is used whenever the pumps need to be stopped in the middle of a gradient, flush, idle or priming operation.

7.8.7 BATCHES

Allows the user to change identification information on the solution batches. This is used, for instance, when a batch of one solution is exhausted and another batch is brought in to replace it.

7.8.8 QUIT

Returns to the Top Menu.

7.9 GRADIENT_DEFINITION

This option allows the manager of gel production to define a gradient. Each gradient definition can be named and stored for later recall. Once the gradient has been set up on the screen, QUITting the GRADIENT_DEFINITION option causes the necessary pump commands to be sent to the pump controller: this takes about a minute (the indicator at the top right of the screen will say "SEND"). Wait for this to complete, and then proceed to the next option (generally RUN).

```
Y1: [W15] 'R                                                          READY
NEW  RECALL  MODIFY  SAVE  GRAPH  PRINT  QUIT
Create a new gradient specification or modify the current one
          Y         Z        AA        AB        AC        AD        AE    AF
1   R
2                  G R A D I E N T    D E F I N I T I O N
3   ------------------------------------------------------------------------
4   Gradient Name: L11-18   Date: 20-Jun-91          Author:  L Anderson
5   ------------------------------------------------------------------------
6   Comment:       LSB standard 11-18%T gradient using v1.08 4-sol'n Angeliq
7   ------------------------------------------------------------------------
8                                                  GRADIENT SPECIFICATION:
9          Volume:  2050 ml                           Position       %T
10      Pump Speed:   400 ml/min                  ---------------------------
11                                                     Top          0     11.0%
12       L/H Pers:  1.77                                            5     11.0%
13       L/H Temed: 15.91
14
15     Acryl stock:  28.0%T acrylamide          Bottom       100     18.0%
16                                               ---------------------------
17        Duration:   5.1 minutes                      Min. %T:      0.00%
18      Buffer var:   2.2% (Top->Bottom)               Max. %T:     20.89%
19    Buffer stock:   6.0 X desired gel conc.          Span %T:     20.89%
20
24-Jun-91  05:10 PM                        CMD
```

The screen shows :

- identifying information (Name, Date of entry, Author and a Comment describing the gradient),
- the gradient specification itself,
- the desired total volume to pump,
- pump speed,
- acrylamide stock solution concentrations,

- the expected duration of the run,
- the variation in buffer concentration from the top to the bottom of the gel (due to differences in the light and heavy pumps),
- and the concentration required in the buffer stock to give the required final gel concentration.

Options are:

7.9.1 NEW

Set up a new gradient definition from scratch. This option erases all the existing values on the screen and then prompts the user for the required information, step by step.

7.9.2 RECALL

Recall a previously entered gradient from a disk file. The disk files result from SAVEing a gradient definition (see 7.9.4). A gradient named "TEST5" would be stored in a file named TEST5.WK1 in a directory called C:\ANGEL\GRADIENT\

Choose a file to recall from the list presented underneath the menu bar at the top of the screen. A choice is made by moving the bright cursor bar with the left and right arrow keys until it lies over the desired choice: then hit [Return]. The choices are listed alphabetically. Remember that if the choice list extends to the right of the screen there may be more choices than you can see: to see the rest just keep moving the cursor bar to the right.

7.9.3 MODIFY

Allows you to change selected parameters listed on the screen.

MODIFY options are:

7.9.3.1 VOLUME

Set total volume to be pumped to the casting box by the RUN_GRADIENT command.

7.9.3.2 SPEED

Set the speed of pumping (both channels combined). As the pumps generate significant pressures at high flow rates, limit the speed to 400 ml/min or less. In general, a slower flow rate will give better gradients, due to decreased mixing on arrival in the box.

7.9.3.3 ACRYL_STOCK

Allows entry of the concentration (expressed as a weight/volume percent) of acrylamide monomers (acrylamide plus crosslinker, e.g., bis) in the stock solution.

7.9.3.4 NAME

A unique name identifying the gradient. This name will be used to generate the file name (when the gradient is saved) and will be recorded in the log file whenever this gradient is used in preparing gels. The name is limited to six characters.

7.9.3.5 AUTHOR

The name or initials of the person defining the gradient.

7.9.3.6 GRADIENT

Specify the parameters defining the gradient shape, in terms of percent acrylamide (%T) as a function of position in the gradient.

```
X1: [W15] 'R                                                          READY
Top  Bottom  1st  2nd  3rd  HELP  QUIT
Specify %T at the top of the gradient (height = 0)
          Y         Z        AA        AB        AC        AD        AE    AF
1   R
2                  G R A D I E N T    D E F I N I T I O N
3   ------------------------------------------------------------------------
4   Gradient Name: L11-18   Date: 20-Jun-91          Author:  L Anderson
5   ------------------------------------------------------------------------
6   Comment:       LSB standard 11-18%T gradient using v1.08 4-sol'n Angeliq
7   ------------------------------------------------------------------------
8                                                  GRADIENT SPECIFICATION:
9          Volume:  2050 ml                           Position        %T
10      Pump Speed:   400 ml/min                  --------------------------
11                                                    Top        0     11.0%
12        L/H Pers:  1.77                                        5     11.0%
13       L/H Temed: 15.91
14
15     Acryl stock:  28.0%T acrylamide          Bottom       100     18.0%
16                                               --------------------------
17        Duration:   5.1 minutes                      Min. %T:      0.00%
18      Buffer var:   2.2% (Top->Bottom)               Max. %T:     20.89%
19    Buffer stock:   6.0 X desired gel conc.          Span %T:     20.89%
20
24-Jun-91  05:11 PM                        CMD
```

Values must be given for the %T at the TOP and BOTTOM of the gradient. If no other values are given, then the gradient will be linear from top to bottom. If the TOP and BOTTOM %T values are the same, then a homogeneous (non-gradient) gel will result.

In order to enter more complex gradient shapes, the user can enter three additional points between the TOP and BOTTOM. Each point is specified by position in the gradient (0 being the TOP and 100 the BOTTOM) and %T desired at that position (given in percent). Obviously the positions cannot be <0 or >100, nor can any of the %T values be less than the Min%T shown or greater than the Max%T (these are the calculated minimum and maximum values that can be achieved using the light and heavy stock solutions you've specified). In addition, the positions given have to be in uniformly increasing order (i.e., like 0 - 10 - 45 - 100, not like 0 - 10 - 5 - 100), and the %T must increase throughout the gradient (if it didn't the density gradient would be upset).

7.9.3.7 COMMENT

A descriptive comment designed to allow one to remember what this gradient is to be used for.

7.9.3.8 QUIT

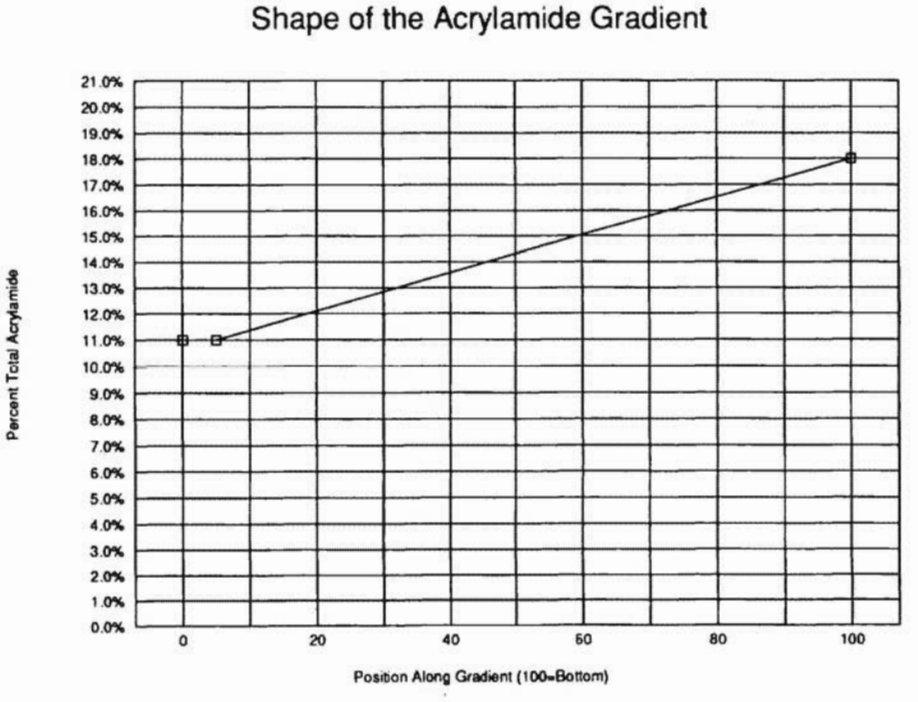
Return to the GRADIENT_DEFINITION menu.

7.9.4 SAVE

Save the gradient as a disk file for future recall. The gradients are saved as small Lotus 1-2-3 spreadsheets and therefore have the form "name.WK1".

7.9.5 GRAPH

Produce a graph on the screen of the shape of the gradient being defined. Hit any key to return to the GRADIENT_DEFINITION screen.



7.9.6 PRINT

Print out the gradient definition as displayed on the screen (assuming you've connected a printer to the PC and set up Lotus 1-2-3 to use it).

7.9.7 QUIT

Return to the Top Menu.

7.10 PUMP_CALIBRATION

Due to the changes that occur in the tubing of the peristaltic pumps with age, it is advisable to periodically calibrate the pumps. To perform this calibration, one wants to measure the amount of liquid delivered by each of the four modules in each of the two pump stacks (HEAVY and LIGHT). This is done by pumping water from weighed containers and then re-weighing the reservoirs to establish the weight (and hence volume) of water pumped out by each module.

```
AJ1: [W12]                                                          READY
DRIVE  ENTER_WEIGHTS  TUBE  STEPS  RECORD  HISTORY  PRINT  QUIT
Drive the pumps the required number of steps prior to weighing
        AJ        AK      AL      AM      AN      AO      AP      AQ       AR
1
2            P E R I S T A L T I C   P U M P   C A L I B R A T I O N
3   ------------------------------------------------------------------------
4              Number of motor steps to run: 42,000 steps
5              Volume of the delivery tube:   68.00 ml
6                                                        Date:   13-Jun-91
7
8
9
10  Channel:     <------ LIGHT ------><------ HEAVY ------>  LIGHT   HEAVY
11                Begin   End   Taken  Begin   End   Taken  Percent  Percent
12
13  Bottom (big) 236.3  166.3   70.0  332.0  262.1   69.9    66.8%    74.6%
14  Top (Quick)  320.1  303.1   17.0  303.1  288.5   14.6    16.2%    15.6%
15  3rd from top 344.5  335.6    8.9  356.1  351.2    4.9     8.5%     5.2%
16  2nd from top   0.0    0.0    8.9  335.6  331.3    4.3     8.5%     4.6%
17
18  Total                     104.8                 93.7   100.0%   100.0%
19  ml/step:                 0.0025               0.0022
20
24-Jun-91  05:09 PM                      CMD       CALC
```

The screen shows the volumes pumped by each of the eight modules, plus the percentages of the total delivery calculated from the entered weights. The number of pump steps driven is shown, as is the volume of the delivery tube (between the lucite mixing manifold and the end of the tube that sticks into the casting box grommet). Options are:

7.10.1 DRIVE

Move the pumps the required number of steps (e.g., 21,000 steps = 10.5 ml) in order to remove liquid from the pre-weighed reservoirs. Do this once per set of measurements, i.e., move the pumps once and then weigh.

7.10.2 ENTER_WEIGHTS

Enter the weights of the liquid removed from the weighed reservoirs by one DRIVE operation. The typical procedure is to enter the four appropriate beginning weights for the water-filled vessels (Light; Begin; Bottom (big), Top (Buffer), 3rd from top, 2nd from top), then Drive the Light pumps to remove liquid using the four respective Light channels, and re-weigh the vessels and enter the weights as the Light; End values. You may then select Light; End; Copy to copy these values for use as the Heavy; Begin values if you re-use the same vessels (without adding more water). Otherwise enter the Heavy: Begin values one by one. Finally Drive the Heavy pumps, re-weigh the vessels and enter the Heavy; End weights. The system will now calculate the weights of water removed from the weighed vessels by each individual pump, and use this information to calibrate itself for gradient operation.

Note that two of the light pumps (2nd and 3rd from top) are connected, and feed from the same tube. Hence only one measurement will be made for the sum of these two pumps.

Select option RECORD to record the calibration operation just completed.

7.10.3 TUBE

Enter the volume of the delivery tube carrying liquid from the mixer to the casting box. This volume needs to be known in order to take the tube's contents into account in producing the correct gradient.

7.10.4 STEPS

Enter the number of pump steps to be moved in the DRIVE option. One pump revolution typically equals 2000 steps. This value is usually left constant (it is used only in the calibration step).

7.10.5 RECORD

Record the calibration in a table showing the last ten calibrations. This "history" table allows you to see how the pumps are changing over time (see below).

7.10.6 HISTORY

Look at graphs of the change in pump calibrations over time. You can select either the LIGHT or HEAVY sets of pumps in the secondary menu that appears. Hit any key to return from looking at the graph.

7.10.7 PRINT

Print out the pump calibration as shown on the screen (assuming you've connected a printer to the PC and set up Lotus 1-2-3 to use it)

7.10.8 QUIT

Return to the Top Menu.

7.11 CHANGE_LOG

Each gradient pumped results in the writing of a brief record of the gradient parameters, solution batches, operator and gel batch identification to a log file on the PC's hard disk. It is convenient to start a new file periodically (monthly, for instance) so that a reasonably-sized group of records is stored in each log file. One method of naming these files is to use the month and year: **AUG91.LOG** would cover operation during August of 1991, to be followed by **SEP91.LOG**, etc. The CHANGE_LOG option prompts the user for a new logfile name and causes subsequent gradient runs to be recorded there. If no file is given, a file named DEFAULT.LOG is used.

7.12 Problems

If you get stuck at some point, and are sure that the system is not just in the middle of recalculating or sending data to the pumps (sometimes

requiring about a minute), then you may need to restart from the Top Menu. This can be done by holding down both the Ctrl and Alt keys and then pressing R.

If the small red LED mentioned in Section 7.7 remains lit after executing SETUP, then the pump motor controller has perceived a problem. If this occurs, turn the pump controller power off and then back on (using the line cord switch) and retry SETUP.

7.13 Record Keeping

Aside from the printouts of GRADIENT_DEFINITION and PUMP_CALIBRATION screens, there are two other operational records of interest. The first is the "HISTORY" table describing the last ten pump calibration results. This table is printed out by selecting PRINT in the HISTORY option itself.

The second is the gradient production log. This is a separate system file containing several lines for each gradient run made. The option CHANGE_LOG in the Top Menu allows you to start a fresh log file periodically. In order to see what is in the log, you can either type:

C:> **type angel\aug91.log** Return to see the file contents on the screen, or

C:> **print angel\aug91.log** Return to have the file contents printed on a printer, substituting in either case the correct file name in place of aug91.log.

7.14 Suggested Recipes

The following stock solution recipes have proven useful at LSB. Adjustment may be necessary in order to optimize polymerization times.

You may wish to use different stock acrylamide concentrations to achieve different gradient ranges; the GRADIENT_DEFINITION screen allows you to alter the stock concentrations and see what range of gel concentrations can be produced with those stocks. Whatever alterations are made to the acrylamide stocks, remember that it should always contain about 13% glycerol (for density).

Use these solutions at room temperature removing them from the refrigerator some time before use. You may put the bigger stock solutions (buffer and acrylamide) in a warm waterbath, or in the sink half filled with lukewarm water.

Degas the buffer, and heavy and light acrylamide solutions before using them. Use a high vacuum pump and the amber plastic bell jar (**26**). The solutions are degassed when bubbles appear like water boiling.

CATALYST and INITIATOR: Prepare fresh persulfate every week or two.

TEMED	0.075%	1.5ml / 2,000ml
Persulfate	1.3%	6.5g / 500ml

ACRYLAMIDE (28.0%T Stock):

Acrylamide solution (30.8%T), e.g. Protogel		2,000ml
Glycerol	9%	200ml

Directions: Never mouth pipette acrylamide solutions and wear gloves when handling them. Acrylamide is a NEUROTOXIN.

---

26) Obtained from Cole-Parmer as a 4.5 gal PEI jar (L-06761-30) and a vacuum base plate (L-06763-02). This setup will hold three 2-liter Corning plastic roller bottles used as solution reservoirs.

BUFFER

It is often convenient to use Sigma's Tris pre-mix in order to avoid having to adjust the pH of the buffer. We use Trizma -8.1 to achieve the pH desired at 10°C. The recipe should be made to yield a final buffer concentration of 0.375M in the gel: the dilution factor introduced by the pumps (which is calculated from the pump calibrations and displayed on the GRADIENT_DEFINITION screen) is assumed here to be 6.0. Hence the buffer stock concentration is 0.375 x 6.0 = 2.25M. Since the Tris premix comes in 500g bottles, we make a volume of solution that uses this amount.

Tris premix (Sigma Trizma -8.1)	1.2375M	500g	to 1,589 ml with pure water

7.15 Modifying the Lotus 1-2-3 Configuration

7.15.1 Used with Lotus 2.0

If you change the drivers in Lotus (by adding a new printer driver, for example), then you may find that the Measure screen does not come up after 1-2-3 startup (as it should). This happens because the RS232 driver "falls out" if another driver is subsequently added. The necessary RS232 driver can be easily reinstalled by doing the following from the 1-2-3 directory:

C:\123> **NEWLIB 123.SET RS232.DRV -u**

This adds the serial driver (for RS232 communications over the serial line to the pump controller) back to the Lotus 1-2-3 driver set. The Measure logo should once again come up whenever you start Lotus 1-2-3.

7.15.2 Used with Lotus 2.2

Since the Measure software is loaded directly by Lotus 1-2-3, it is not necessary to bother about reloading the driver if changes are made.

7.16 Condensed Dalt Instructions (using Angelique)

- Fill the Dalt tank to the appropriate height with purified water.
- Check to see that the coolant circulation is on.
- Turn the buffer circulating pump on once it is filled with water and check for flow.
- Weigh out the correct SDS running buffer mixture for this volume (see recipe section) and add the dry powder directly into the center chamber.
- Once the buffer is dissolved, make sure to raise the two barrier combs (at the same time) so as to mix the total Dalt tank contents with the dissolved center chamber. Lower the combs; the tank is ready to run.

- Remove the stock solutions from the refrigerator. Warm the acrylamide and buffer solutions with a waterbath, and degas them.
- Fill the gel casting box with cassettes interspersed with a total of 2 or 3 appropriately-sized sheets of "bubble-pack" material and a sheet of polycarbonate between each cassette.
- Prepare gel labels (section 17) and drop them into the cassettes on the side opposite the gradient inlet port.

- Place the Equalizer™ on one side on the top of the casting box, assigning one needle into each cassette.
- Turn on Angelique (both computer and pump controller). At the DOS prompt,"C:>", type what appears below in **bold** at the appropriate times:

C:> **CD\123** Return
C:> **123** Return
q Return
/FRANGEL Return

- Select the SET_UP option to set up the pump controller for correct operation (this step is required every time the power to the pump controller unit is first turned on).

• Choose the GRADIENT_DEFINITION option and either create, recall or modify the gradient information to get the desired design. Exit using the QUIT option: this will load the pump controller with the gradient information.

• Choose RUN from the Top Menu.

• Place the five intake tubes in the correct solution reservoirs and place the delivery tube in a waste container (to receive polymerizable acrylamide).

• PRIME the pumps, making sure that all air bubbles are purged from the pumps and the delivery tube.

• FLUSH the delivery tube in preparation for the gradient (the delivery tube is still in the waste reservoir).

• Place the delivery tube in the casting box grommet, load the side chamber with dense solution, and use GRADIENT to pump the gradient into the box.

••• Repeat the FLUSH, GRADIENT, FLUSH, GRADIENT, FLUSH..... cycle as required for the number of casting boxes to be filled.

• Fill each of the syringes of the Equalizer with sec-Butanol, buffer saturated. Let this overlay solution drop onto the top surface of the acrylamide. Check that all the syringes drop their contents.

• Finally, place the intake tubes in a reservoir of water and use PRIME to rinse all the acrylamide and other solutions out of the system.

• Allow gels to polymerize for at least 1 hour.

• When polymerization is complete, remove the front of the casting box. The dense displacing solution will leak out at this point into the tray beneath the box.

• Carefully unload the cassettes from the box using a single-edged razor blade to separate the plates.

• Wash each cassette carefully with water to remove acrylamide adhered to the outer surfaces.

• Place the plates in alphabetical order in dishracks (the dishracks should be in plastic containers with 1/4 of an inch of water).

• Place the Dalt gels in position with the rubber hinge strip to the right and the label readable from the front.

• Extrude the Iso gel directly onto one of the cassette top edges (so that it is extended along it with the acid end to the left) with the aid of water pressure provided by a small syringe. To make a suitable syringe gadget, cut the large end off a micropipette tip such that it will fit tightly on the Luer nipple of a 1 ml disposable plastic syringe. The syringe is filled with water, the tip inserted into the top end of a gel tube and the gel extruded by means of water pressure generated by pressing downwards on the syringe plunger. Pressure should be the minimum required

• Roll the rod gel over the edge of the Dalt cassette with a squeeze bottle containing Iso equilibration buffer. Use this buffer to help lubricate the Iso. Smooth the gel into position with a spatula, eliminating any air bubbles underneath (between Iso and Dalt gels).

• Place a Wedgie into the loading cassette slot (don't push too hard but enough to hold the Iso against the Dalt gel).

• Insert the cassette carefully into the Dalt tank, with the Iso gel along the left side and with the rubber cassette hinge along the bottom.

• Place a "buffer raiser level" into the tank, like a gallon plastic bottle filled with water.

• Close the lid on the tank. Attach the electrodes to make proper electrical contact with the power supply.

• Turn on the power supply and set a low voltage (≤50 volts) for running the gels.

• As each Dalt gel is ready to load, turn off the power supply, open the tank and insert the cassette. Then close the tank and restart the power supply while you prepare the next cassette.

• Remove the "buffer level raiser" once about half (10) of the gels have been loaded.

• After loading the last cassette, increase the voltage to the desired run value in the power supply and run the gels until the blue tracking dye reaches the right side of the gel holder (the "bottom" of the gel).

- When you are ready to remove the cassettes, turn off the power supply first.

- Remove the cassettes carefully, one at a time, using the fingers of both hands, and place them in dishracks (10 per rack) standing in containers with 1/4 in. of water.

- Carefully remove the Wedgies™ from the cassettes, rinse with abundant water and place them to dry.

- Open each cassette carefully, making sure that the gel comes with one of the plates and is not sticking partly to both (the gel could tear).

- Use a razor blade to separate the gel from the spacers (if it lies on the plate with spacers attached) and run it along the gel bottom (between gel and plate) in either case.

- Peel the gel from the glass carefully, lifting it by the bottom (high %T end) and place it in a box of staining solution.

8 Detecting Proteins on Gels

8.1 The Virtues of Coomassie Blue

Coomassie Brilliant Blue R-250 (CB; Color Index #42660) is a synthetic dye originally made for use in coloring wool fabrics. It binds strongly to proteins, is highly colored and provides a sensitive, fairly linear detection system for most polypeptides. ICI, Ltd., the original manufacturer, has ceased production. Today, the material provided by various chemical houses may differ widely in purity. It is always advisable to filter the stain solution before use.

• After unloading the gels from the cassettes, put ten gels into 1.5 l of fixative solution (1 l for the 7"x7" gels) in a plastic box with tightly-fitting lid (e.g., RubberMaid or Tupperware). The box should be at least .5" larger than the gels in planar dimensions, and 1.5" deep. The fixative solution is made up of 2% phosphoric acid, 50% ethanol, and 48% tap water. We no longer use an acetic acid-ethanol stain because the stain had to be mixed just before use to prevent formation of ethyl acetate. Methanol, while a good gel fixative, is inherently more dangerous than ethanol. The use of "denatured" ethanol is to be avoided since it adversely affects staining.

• Fix the gels for at least 3 hours (or overnight) on a reciprocal shaker. It pays to take some care in setting the shaker so that the gels are agitated enough to prevent adherence to one another but not enough to cause gel crumbling.

Since gels are easily damaged in the process of removing one solution from the box to add another, it has proven useful to make a simple tool to keep the gels flat on the bottom of the box while it is inverted to drain. A square of thin (1/4") acrylic plate is cut so as to just fit inside the box, and a short section of thick (~1") acrylic rod or tube is glued to middle of one surface as a handle. This paddle is placed into the box and pressed down gently to immobilize the gels; then the box can be inverted to drain out the liquid contents. Fresh solution can then be poured onto the back of the paddle (once again preventing damage to the gels), and the paddle removed.

• Wash the gels three times into (2 l for each box) of distilled tap water for about 30 minutes.

• To stain, put the washed gels into 1.5 l of Neuhoff Concentrate (see recipe section). After 1 hour, add ~1 gr of Coomassie Blue. The characteristic of the Neuhoff solution is that only part of the stain powder dissolves, enough to stain the proteins in the gels but minimizing the background. The protein spots start to be visible after a few hours. Leave the gels shaking in this stain solution between 4 and 8 days for complete stain.

• After staining, carefully wash the gels, one by one, with tap water using a sink hand sprayer attachment. To turn over and handle the gels it has proven useful to make another simple tool. Make two plastic frames, each with an inner open surface area of 8"x10", and hinge them together along one side to create a book. Stretch a close-knit plastic mesh across each frame and glue it in place. A gel is placed on the mesh surface of the open "book" and rinsed with water. The book is then closed, turned over and opened to rinse the other side of the gel. Spray gently to avoid breaking the gel. Once washed, the gel is ready to be photographed or scanned.

• Store the gels in ~0.2% sodium azide if you want to save them, as the gels often become moldy if stored in plain water for an extended period.

8.1.1 Photography of Coomassie Blue-Stained Gels

Stained gels should be photographed through a yellow filter on panchromatic film in order to maximize spot contrast. A variety of film formats can be used, from 35 mm to 4"x5" sheet film. We recommend the use of 70 mm roll film as a good compromise.

A useful setup should include a good light box (whose illumination varies very little from center to edge) and, if possible, a camera with a motor drive and remote cable release button. This allows you to work alone with wet hands (handling the gels) and at the same time not get the camera wet.

Each frame should contain not only the image of a gel, but also the gel number (the gel number on filter paper in the gel itself is not always easily read on the resulting print). This can be done by writing gel numbers on Scotch tape strips, which are laid beside the appropriate gel during photography. A somewhat better approach is to make up a column of gel identifiers (the letters a-w in the numbering scheme used here) on a

transparent sheet at the edge of the light box frame and use a movable arrow to point to the correct letter for each shot. The gel batch is identified on a piece of clear tape also in the picture (e.g., A31).

It is also useful to include a neutral density wedge if later image analysis may be attempted from the negative or print.

8.2 Silver Staining

Many methods of silver staining have been devised. Because different procedures seem to work better in different labs (presumably on account of water and reagent differences), it is worth trying several. A major factor regardless of the particular method is the use of very fresh and pure chemicals and solutions. All water must be very pure. The use of house distilled water often produces a somewhat cloudy background. The Dalt tank must be very clean and must have fresh buffer. Keep all solutions covered so that dust does not fall into them. Make sure that there are no fingerprints or dust on the Dalt cassettes. The method described here is based on that of Guevara et al.(**27**), as modified by Sharron Nance.

Best results are obtained if gels are not first stained with Coomassie Blue, as this may make backgrounds darker; it is possible, however, to silver-stain gels that have previously been stained with Coomassie Blue.

Use gloves for all steps including the unloading of the Dalt cassettes. Shake the gels during each step. Allow 200 ml of solution for each 7"x7" x 1.5 mm gel.

8.2.1 Fixation

• Fix freshly run Dalt gels (five gels to a box) for at least 4 hours in 1 l of either of the following freshly made solutions. We have had success with both. Perhaps you should try both fixatives to see which one works best with your samples.

A: 50% ethanol / 0.1% formaldehyde (**28**).

B: 2.5% sulfosalicylic acid / 5% acetic acid in 20% ethanol.

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27) Electrophoresis 3, 197-205, 1982.

28) Some methods specify that gels be fixed in glutaraldehyde but often backgrounds are too high when gels are fixed by this method. Surface contaminants or dry gel surfaces also increase the background.

• Drain gels after at least 4 hours of fixing and put them into 20% ethanol overnight.

• Rinse the gels the next morning in 20% ethanol for at least 1 hour (3 changes for $\geq$ 20 min each).

8.2.2 Silver Diamine Staining

• It is very important to make this solution in the following order:

740 ml very pure water

200 ml ethanol. Mix the water and ethanol and wait for the result to equilibrate to room temperature before proceeding.

2.35 ml 6N NaOH (or 3.53 ml 4N NaOH)

10.5 ml NH_4OH (add just before use). The stock NH_4OH concentration is 60%. The bottle used should be fresh and it should be kept tightly capped except for rapid and infrequent removals, on account of volatility.

• Immediately before use, slowly add 5 g $AgNO_3$ dissolved in 50 ml water to the above solution while stirring vigorously. A transient brown precipitate will appear as the $AgNO_3$ is added.

• Shake gels in this solution for exactly 1 hour.

8.2.3 Washing

• Rinse briefly with pure water.

• Wash exactly 1 hour with 20% ethanol (20 minutes per wash, three washes).

8.2.4 Development

• Mix in order:

800 ml water

200 ml ethanol. Mix the water and ethanol and wait for the result to equilibrate to room temperature before proceeding.

50 mg citric acid. Citric acid may be added as a freshly weighed solid or, if convenient, maintained as a stock 100 mg/ml solution in water.

0.5 ml formaldehyde (add immediately before use). Use a fresh bottle and keep tightly capped.

• Shake gels in this solution until the low molecular weight spots are developed. Then pour off the solution. The development step is temperature sensitive. A simple rule of thumb for the time it seems to take for the developing process is 50 minutes minus the temperature of the lab. (If the temperature of the lab is 25°C, 50 - 25 = 25 minutes developing time.) Note: Occasionally a slightly altered concentration of either citric acid or formaldehyde will give better results.

8.2.5 Stop

• Mix 5 ml glacial acetic acid in 1 l water and put the solution on the gels while the gels are shaking; continue shaking them only about 1 minute.

8.2.6 Wash

• Rinse the gels (3-4 times) with pure water for 45-60 minutes.

• Shake the gels overnight in 20% ethanol to prepare them for photography. Then store the gels in 20% ethanol (changed daily) until photography is completed.

8.2.7 Photographic Recording of Silver-Stained Gels on XRD Film

This procedure (**29**) outlines the method for making positive black and white transparency image transfers from silver-stained gels onto Kodak X-Omat Duplicating Film (DUP, Cat. # 163 7842). Protein spots come out black in these images regardless of their color in the silver-stained gel. The general procedure assumes a bit of basic photographic knowledge and is, in outline, as follows: The wet two-dimensional slab gels are sandwiched between two thin, transparent supports (e.g., Kodak Roller Transport Cleaning Sheets or clear acetate), and then placed onto an 8 x 10 inch sheet

29) Harrison, Harold H., Clin. Chem. 29(8): 1566-1567, 1983.

of X-Omat Duplicating Film. A white or blue light exposure is then made and the film is developed. The result is a positive image transfer in an essentially 1:1 scale "contact print" format.

The materials you will need to produce XRD images are: (1) an adequate supply of X-Omat DUP film, (2) your silver-stained gels, (3) a box of fresh water into which you will transfer gels after copying, (4) five or six roller transport clean-up sheets (Kodak # 4955) 8 x 10 inches or 11 x 14 inches, (5) access to an X-Omat or similar X-ray film processor, and (6) an indelible felt-tip marking pen. A flashlight might also come in handy for reading the gel number labels. Plan on 2-3 hours work per batch of 20 gels.

Exposure guide: Typical exposure times are 20-30 seconds at 120-150 W and 50-90 cm enlarger height. Factors affecting development are primarily related to gel background stain, but there are also batch-to-batch variances due to X-Omat chemical status and film sensitivities.

- Run a few test shots of an average gel from your batch.

- Immediately after the exposure, feed the film into an X-Omat and compare the developed film image with the original gel to determine if the exposure was satisfactory. Due to gel-to-gel staining differences, you may need to validate each image individually, rather than to rely on a uniform setting as with Coomassie Blue stain photography. On each XRD image record the gel number.

- After you have finished with each gel, transfer the gel to a "finished" water box. Dry the cleaner sheets before the next gels are placed on them. The cleaner sheets tend to pick up some of the silver stain after a number of runs, so you should inspect each one before using it again and discard the sheet if it is badly stained.

8.3 Autoradiography

In many instances, samples must be radioactively labeled to increase the sensitivity of the two-dimensional analysis, or to allow measurement of synthetic rates. The two-dimensional ISO-DALT® procedure for labeled samples is the same as for unlabeled samples. Gels are fixed and stained in the Coomassie Blue stain and destained through the first three steps as described earlier. Next, the gels are soaked in two changes of 2% glycerol in water for 30 minutes (the glycerol solution prevents the gels from

cracking when they are dried). The gels are then ready to be dried using a typical vacuum/heat gel drier (e.g., Hoefer SE 1160), following the manufacturer's instructions.

It is helpful to label each dried gel with the appropriate gel number in radioactive ink made by adding 10 µCi ^{14}C-glucose/100 ml ink (the specific activity of the glucose is 6 µCi/g). Using a steel drawing pen (the type that you dip in ink every few characters), you can write directly on a corner of the dried gel.

The dried gel can be inserted directly between the film and the loose paper of Kodak "ready-paks" (XAR-2). Five to ten ready-pak films can be placed in a cardboard Kodak exposure holder of the next larger film size.

For large numbers of autoradiographic exposures, it is convenient to build a "gel squasher". To do this a box is built with heavy plywood shelves spaced about 1" apart. A square, closed, flat vinyl bag with one tubing fitting is placed on each shelf and connected to a source of air at about 2-3 psi via a 2-position valve (allowing release of the air as well as pressurization). The small plastic Luer valves used on chromatography columns will do. Place each exposure holder in an empty slot of the gel squasher and open the valve attached to its air bag. Exposure holder and contents should be squashed flat (3psi over one 7"x7" gel is 150 lb. of squashing force, better than bulldog clips).

Record appropriate information in an exposure log book (date, time in, time out, gel numbers, and initials).

8.4 Fluorography

This procedure is used when the most sensitive detection of radioactive label is required, i.e., when the sample has been radioactively labeled with ^{3}H, ^{14}C, or low levels of ^{35}S. Run gels as usual and destain them, draining off final destaining solution completely.

8.4.1 Impregnating the Gels with Scintillant

• Put gels (five to a box, maximum) into dimethyl sulfoxide (DMSO I) at 250 ml/gel, and shake for 30 minutes. Return the DMSO I to the original DMSO I container. DMSO I should be labeled "waste" after the third use.

• Put gels into DMSO II (250 ml/gel), and shake for 30 minutes. Return DMSO II to the original container. DMSO II should be labeled "DMSO I" after the third use.

• Put gels in 13% 2,5-diphenyloxazole (PPO) made up in DMSO. Shake for 3 hours. Return PPO solution to the original container. This solution can be used eight times. After that, the PPO must be recovered and the DMSO discarded.

8.4.2 Precipitating the Scintillant and Drying the Gels

• Put the gels into distilled water (250 ml/gel), and shake for 15 minutes.

• Discard the water.

• Put the gels in 2% glycerol (250 ml/gel), and shake for 15 minutes.

• Change the glycerol, and shake the gels an additional 15 minutes.

• Dry gels on a gel dryer.

8.4.3 Exposure

• Put gels on XAR-2 film that has been flashed (exposed to a quick flash of light to expose the film sufficiently to linearize the photographic response: about 0.15 absorbance units of background).

• Place gels on preflashed film in a container that holds them tightly together. One means of doing this is to place gels and films in a thick black plastic bag, evacuate the bag, and heat seal it closed. Alternatively, use Kodak X-Omatic (or equivalent) x-ray cassettes or the traditional flat acrylic plates squashed with bulldog clips.

• Store the gel/film sandwiches at -80°C throughout the exposure.

• Thaw the gels and develop the film in an X-Omat.

9 Western Transfers

This technique is based on a modification (**30**) of the original nitrocellulose electroblotting method (**31**). The main difference with other techniques is that the procedure described here uses previously stained gels. We use this approach so that we know the pattern to be transfered is a good one, and so that, under certain circumstances, part of the Coomassie blue stain pattern transfers to the membrane along with the proteins, giving rise to an already stained blot.

Two kinds of membranes may be used: nitrocellulose or polyvinylidine difluoride (PVDF). The protocols in using these two membranes differ because methanol causes shrinkage (or dissolution) of nitrocellulose.

The Dalt tank is filled with the same buffer used to perform the second dimension electrophoresis (Dalt tank buffer) which can be buffer left over from a previous second dimension run (this is both convenient and economical). Although the blotted proteins have been denatured in SDS, the membrane-bound molecules still react with appropriate specific antisera even after storage of the transfer in air at room temperature for several months.

When used as transfer tanks, the 20-place Dalt tank holds ten gels (five each in two transfer assembly racks, Hoefer's ID 530: Figure 9.6.1); the 10-place Dalt tank holds 5 gels. The transfer assembly consists of two sheets of plastic "egg crate" lattice material, between which a sandwich of Scotchbrite sponge/filter paper/membrane/gel/filter paper/Scotchbrite sponge (**32**) is compressed by means of plastic clips (Figure 9.6.2). A gel is placed on the cathodic side of each membrane sheet, the sheet being numbered with India ink for identification. Current is passed through the gel/membrane sandwiches by means of the platinum electrodes mounted on the long side walls of the Dalt tank.

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30) Specific antiserum staining of two-dimensional electrophoretic patterns of human plasma proteins immobilized on nitrocellulose. Anderson, N. Leigh, Nance, Sharron L., Pearson, Terry W., and Anderson, Norman G., Electrophoresis 3: 135-142, 1982.

31) Towbin, H., Staehelin, T. and Gordon, J., Proc. Nat. Acad. Sci. USA 1979, 76, 4350-4354.

32) Scotchbrite is a trademark of 3M.

9.1 Assembling the Gel-Membrane Sandwiches

Wear gloves when handling membranes. The oils of the fingers will prevent proper membrane wetting, and epidermic proteins bind with high affinity to the membrane giving undesirable background fingerprints on staining.

• Since PVDF membranes are very hydrophobic, it is necessary to prewet them by soaking in 100% methanol for 1-3 seconds or in 5% Tween™-20 for about 15 minutes; afterwards wash the membranes in water for 1-2 minutes. Membranes like S&S NC™ nitrocellulose have a wetting agent incorporated and only need pure water to be wet.

• After equilibrating the gel and the membrane in transfer buffer (the Dalt tank buffer) for 10-15 minutes, assemble them in the plastic transfer cassette in the appropriate order (sponge/filter paper/membrane/gel/filter paper/sponge). Care should be taken during assembly of the gel/membrane sandwiches to remove any air bubbles that might appear between the two. This can be done by gently rolling a pipette across the sandwich (like rolling dough with a rolling pin).

9.2 Performing the Transfer

• Insert the cassette in the correct running orientation into the transfer rack slot. Since the proteins are transported by the electric current from the gels to the membrane, the order of the components of the sandwich in the tank must be appropriate for the direction of the flow of current (the gel on the left (-) and the membrane on the right (+)). Note the arrows on the cassette holding clips; these help to prevent misloading and consequent total loss of the proteins.

• For gels made in 7"x7" cassettes, transfer 2-D gel proteins to 6.5"x6.5" membrane sheet in the Dalt tank for about 500 V*hr (about 120 volts for 4 hours). For gels made in 8"x10" cassettes use a sheet of 7.5"x9.5".

• Remove the assembly and discard the Dalt gels. Under most circumstances, the original Coomassie blue image of the stained gel will be visible on the membrane, and can be scanned or photographed for reference.

9.3 Blocking

• Block the transfers with 5% bovine serum albumin (BSA) 20mM Tris buffered saline (TBS), pH 7.4-7.6, for 1 hour at 40°C (or 2 hours at room temperature or overnight at 5°C). Alternative blocking agents include skim milk protein (5% in TBS) and gelatin.

• Pour off blocking solution and save BSA to dilute antisera.

9.4 Reaction with First and Second Antibodies (Immunostaining)

• Dilute (1:100 dilution or greater, depending on Ab) primary antibody (Ab) in Ab incubation solution (1% BSA, 0.05% Tween™-20 in TBS) and pour over the transfer. At least 50 ml is required for each transfer for rocking on the shaker.

• Leave the transfer on the shaker for about 1 hour, depending on the strength of the Ab.

• Pour off Ab, saving and freezing it at -20°C for future use, if desired.

• Rinse each transfer five times with 0.1% BSA in TBS to wash off all traces of unbound Ab.

• Add 1:1000 anti-IgG-phosphatase diluted in Ab incubation solution. For example, if the primary Ab was rabbit anti-human, then use goat anti-rabbit IgG-phosphatase. Store concentrated solutions of these conjugates in the -20°C freezer.

• Shake each transfer in this solution for 1 hour.

• Rinse five times with 0.1% BSA in TBS.

9.5 Enzyme-Based Staining

• Mix 10 parts of TBS with one part each of:

– BCIP concentrate solution (5-bromo-4-chloro-3-indolyl-phosphate)

– NBT concentrate solution (Nitroblue tetrazolium)

• Mix the above solutions at room temperature and use immediately. Do not freeze the mixture, as a precipitate will form. The final solution should be clear and pale yellow.

• Mix 10 parts of TBS with one part each of:

– BCIP concentrate solution (5-bromo-4-chloro-3-indolyl-phosphate)

– NBT concentrate solution (Nitroblue tetrazolium)

• Mix the above solutions at room temperature and use immediately. Do not freeze the mixture, as a precipitate will form. The final solution

• Pour the mixture over the transfer and rock it until staining is adequate (blue spots with white or light blue background).

• Pour stain solution onto next transfer and rinse stained transfer thoroughly with water.

9.6 Western Transfer Figure

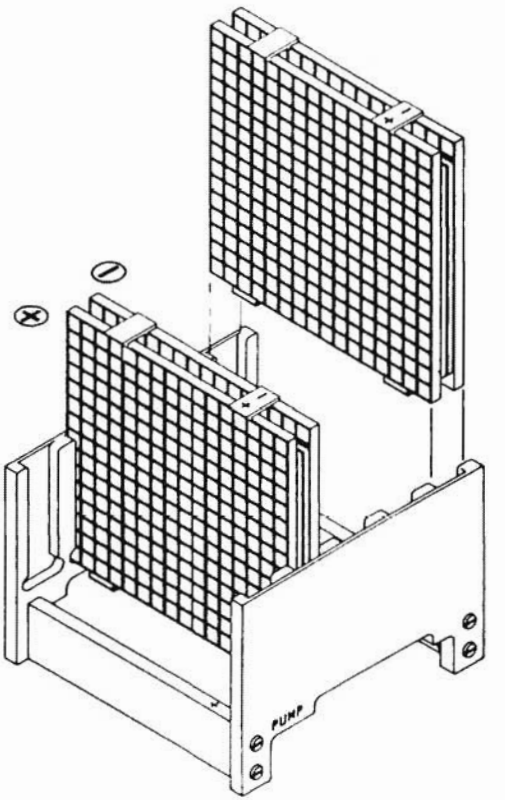

Figure 9.6.1: Hoefer's ID 530 blotting kit: five-place transfer assembly rack with two cassette sandwiches shown.

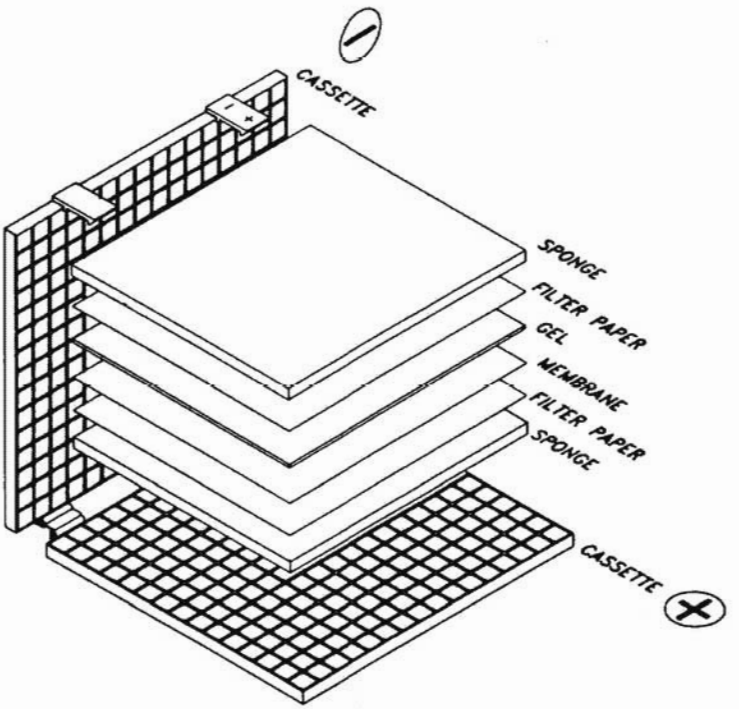

Figure 9.6.2: Exploded diagram of a transfer cassette.

10 Computerized Gel Scanning and Quantitative Analysis

While this document is primarily aimed at communicating gel technology, it must be noted that there is a strong connection between 2-D gels and the field of computer image processing: without computer assistance, the extraction of quantitative data from 2-D gels is impossibly tedious.

10.1 Impact of Detection Method Chosen

To begin with, it is important to realize that all protein detection methods are not equally useful for the generation of quantitative data by image analysis. Despite the fact that radioactive detection methods (autoradiography and fluorography) are widely favored because of their great sensitivity, they do not necessarily provide more useful data because of the increased difficulty in getting the required gel:gel reproducibility. Stain methods (while obviously not suitable for all studies) are enjoying renewed favor partly because of this fact. The following table summarizes the principal characteristics of each widely-used method:

Detection Method	Quantitative Reproducibility	Reasons
Coomassie Blue Stain	Very good	An equilibrium method: although different proteins bind stain in different stoichiometries, the method is reproducible for individually calibrated polypeptides.
Silver Stain	Poor-Very good	A non-equilibrium method: can yield good results if extreme care is taken to standardize staining process.
Autoradiography	Poor-Good	Efficiency of detection is strongly affected by the depth distribution of labeled protein in the gel; this can vary over the gel, giving large variation in detection efficiency over the gel.
Fluorography	Good-Very good	Less sensitive to attenuation than autoradiography. Offers good dynamic range (through multiple exposures), but involves some loss of resolution due to optical spreading.

10.2 Maximizing the Amount of Good Data, Not Simply the "Number of Numbers"

During the initial struggle to get some "real data" from 2-D gels, it is very easy to lose sight of the major purpose of generating quantitative data in the first place: to allow you to demonstrate statistically-significant quantitative effects in your biological system. If you assume that a computer system will automatically convert any set of gels into meaningful data, you may be headed for some disappointment and frustration.

To succeed in building a set of data that will be worth more than a cursory examination, three things must be emphasized: gel quality, gel reproducibility, and experimental design. Using the ISO-DALT® system described in this manual, the first two should be attainable through diligence. The last, experimental design, requires some serious thought about the number of replicates you need for statistics and the size of effect you want to be able to detect. To make these decisions, you need to have available some information on how accurate your measurements are going to be in practice.

It is therefore extremely important, before beginning your "real" experiment, to budget the time to do a few simple studies of quantitative reproducibility in your system. A couple of sets of 10-20 replicate gels of the same one or two samples will tell you fascinating things about the system at hand. If you have CV's (coefficients of variation) for individual spots in the neighborhood of 5-10%, then you will be able to detect quite small changes (10-30%) in these proteins under experimental conditions. If most of the CV's are much higher, then chances are that your effort would be best spent in further optimizing samples and gels before proceeding to quantitative analysis.

In practice, is has been shown that some proteins can be quantitated with very high precision by 2-D: some liver proteins are measurable with CV's of about 5% from gel to gel and even from mouse to mouse, using

Coomassie Blue (**33**,**34**,**35**). Data of roughly comparable quality has been obtained using silver stain and fluorography under tightly-controlled conditions.

In summary, it is often better to have 100 proteins reliably measured than to have 1000 proteins quantitated poorly. In the latter case, measurement errors will produce a large number of potential "effects" (mostly false-positives), and you will spend a great deal of time chasing these down one after another. In the former case, each change that appears statistically valid is likely to be so, and you can proceed with further work based on this information. The additional 900 spots can be added as the experimental program proceeds.

10.3 Scanners

In order to put the 2-D gel image into a computer in the first place, some kind of scanner must be used to convert the picture into digital data (a series of optical density measurements over a grid of points [pixels] covering the image). Several scanner technologies exist capable of digitizing the gel image in preparation for computer analysis. These include vidicon-type (television) cameras, CCD cameras, and laser scanners. In choosing one over another, the main factors are resolution, greyscale discrimination, speed, ability to connect to the computer you want to use, and price.

If you plan to analyze the whole 2-D pattern from each gel, a scanner capable of resolving about 1500x1500 points will probably be required. This is because in the high molecular mass region (top) of the gel, spots as small as 0.3 mm x 0.4 mm can often be found. To measure a spot with any accuracy at all, the computer must have at least three good measurements across it in each direction, hence it must measure these small spots at about 0.1 mm resolution. Given a gel whose "active" area is 15 cm x 15 cm, the

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33) Detection of Heritable Mutations as Quantitative Changes in Protein Expression. Giometti, Carol S., Gemmell, M. Anne, Nance, Sharron L., Tollaksen, Sandra L., and Taylor, John., J. Biol Chem., 262: 12764-12767, 1987.

34) Differences between 2-D electrophoretic protein patterns of livers of male and female mice. Anderson, N. Leigh, Giere, Frederic A., Nance, Sharron L., Gemmell, M. Anne, Tollaksen, Sandra L., and Anderson, Norman G.. In: Proceedings of Biologie Prospective - 6th Colloque International de Pont-à-Mousson, pp 253-260, 1985.

35) Quantitative reproducibility of measurements from Coomassie Blue-stained two-dimensional gels: Analysis of mouse liver protein patterns and a comparison of BALB/c and C57 strains. Anderson, N. Leigh, Nance, Sharron L., Tollaksen, Sandra L., Giere, Frederic A., and Anderson, Norman G., Electrophoresis 6: 592-599, 1985.

computer needs 1500x1500 pixels to describe it accurately. Television-type cameras are not usually able to offer anything like this resolution. Laser scanners usually can, as can many CCD scanners.

The greyscale discrimination needed is usually in the range of 8-10 bits. This means that the scanner can distinguish, respectively, 256 or 1024 shades of density in the image. Less than 8 bits is not very useful. Most scanners at least claim to offer 8 bits, and some can deliver 10 or sometimes 12. Since most computer systems assume they will get 8 bit data, this is the typical figure.

Scanner speed is important only insofar as you value your own time. It turns out to be very tedious to place a gel in a scanner every half-hour all day. Thus, if the gel can be digitized in 3 minutes, so much the better. In any case, always ask for the realistic scan time. Some companies sell 1-D scanners for 2-D use that can take hours per 2-D gel; these are not really very useful.

The question of connectivity is easily asked and answered. First, does a hardware interface exist to connect the scanner in question to the computer you have, or are considering using? Second, does a software driver exist for the appropriate computer operating system and interface? Just make sure the system you are considering fits together.

Prices are coming down, but scanners currently range from $5,000 to $35,000, with TV-type cameras at the bottom, CCD's in the middle and lasers at the top. Most present systems use CCD scanners costing $15-25,000.

10.4 Computers and Software

Several points should be stressed with regard to the general problem of computer-processing of 2-D gels.

First, the amount of raw data involved in whole-gel analysis is large: 2.5-4 megabytes (Mb) per scanned gel. This means that a workable system must have at least 50-100 Mb of hard disk storage to function, and considerably more to function efficiently in analyzing a large experiment or serving several users.

Second, large amounts of data must be processed in a reasonable time. Because a number of steps are involved, the processing power required to do useful work is not trivial. An IBM PC is not sufficient (though for

analyzing a portion of a gel on a limited set of gels, a PC/AT level machine can do some good). You are probably better off from the outset thinking in terms of a "workstation"-type machine: a microVAX, 68020-based (Sun, Apollo or MassComp), or perhaps 80386-based machine. (The numbers here refer to specific microprocessors, Motorola and Intel respectively, that are frequently used as the basis of workstation-class machines.) A workable system should be able to process at least 10 gels a day. The time-consuming image processing portion of this work is often done by the computer running at night.

Third, good graphics capability is required to help you get the most out of the data. This means the system should have a color (preferably 8-plane) display of at least 512x512 resolution but more suitably 1000x800 or above. The graphical user interface is what you will be working with most of the time, and hence constitutes perhaps the most important part of the system. You will want to display both gel images and spotfile images (pictures generated from the reduced data), edit and manipulate spotfiles, and establish and check gel-to-gel matches of spots. For any system, it is wise to request a demonstration showing how an experiment of 60 gels would be analyzed. If the system works with only two gels at a time, such an experiment can be extremely difficult.

Fourth, think about expandability and connectivity. A good system should be upgradable, both in terms of gel processing capacity and system resources (disks, tape backup, graphics, etc.). It should also be capable of connecting to a network for access to additional resources.

Lastly, realize that a software system capable of doing the required image analysis, gel matching, graphics and statistical manipulations is not likely to be cheap (if it's good). Take some time and compare a range of products.

10.5 The Kepler® 2-D Gel Analysis System

With these and other factors in mind, LSB has developed the Kepler gel analysis workstation based on VAX® **(36)** graphics workstation hardware. This system is optimized for analysis of ISO-DALT 2-D gels, and has been used, during its extensive evolution from the original TYCHO

36) VAX is a registered trademark of Digital Equipment Corporation.

system (**37,38,39**), to analyze thousands of gels, generating some of the largest quantitative 2-D databases in existence. The following description, though it refers specifically to Kepler, illustrates components generally required to produce good 2-D data.

The current version Kepler software can handle experiments with hundreds of gels organized into any desired number of groups. The system is composed of several major components:

EXPERIMENT DEFINITION. The user interactively defines the structure of an experiment by identifying control and experimental groups of gels. Definitions can be changed or expanded to allow for later addition of supplemental data.

SCANNING. The system controls scanning of gel images and stores them on disk. Typical scans are carried out at 100 micron resolution (though this is adjustable), producing 8-bit images in the optical density domain with up to 2048x2048 pixels.

IMAGE PROCESSING. Several image processing procedures are provided. Typical images are 1500-2000 pixels on a side, though larger images can be processed subject to availability of disk and memory resources. Standard analysis protocols are provided, so that a user does not need to know about specific image processing steps, though these can be independently applied in special cases.

SPOT MODEL OPTIMIZATION. The system optimizes a 2-D Gaussian representation of the gel pattern, yielding an accurate representation of the pattern as a list of 2-D Gaussian spots (a "spot parameter list"). Partially overlapping spots and overrange regions are taken into account. The parameters of the 2-D models (X and Y position, X and Y half-widths, and amplitude for each spot) are then used for subsequent interactive graphical and quantitative statistical analysis.

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37) Estimation of two-dimensional electrophoretic spot intensities and positions by modeling. Taylor, J., Anderson, N.L., Coulter, B.P., Scandora, A.E., and Anderson, N.G., Electrophoresis '79, B. Radola, ed., W. de Gruyter, Berlin, pp 329-339, 1980.

38) A computerized system for matching and stretching two-dimensional gel patterns represented by parameter lists. Taylor, J., Anderson, N.L., and Anderson, N.G., Electrophoresis '81, Allen and Arnaud, eds., W. de Gruyter, Berlin, pp 383-400, 1981.

39) The TYCHO system for computer analysis of two-dimensional gel electrophoresis patterns. Anderson, N. L., Taylor, J., Scandora, A. E., Coulter, B.P., and Anderson, N. G., Clin. Chem. 27: 1807-1820, 1981.

IMAGE DISPLAY. The user can inspect scanned, processed, or spot model images with a variety of options. A region of interest can be specified for subsequent analyses. The spot model images are generated very rapidly (in real-time) with a procedure that closely approximates the actual gel pattern appearance (a 1000-spot image can be generated in approximately 1 sec). The system thereby achieves a pattern display speed consistent with pleasant interactive use in the pattern manipulation tasks.

MULTI-WINDOW INTERACTION SYSTEM. The main interactive analysis system presents three main displays: foreground, background and montage. The montage is composed of the same region from each of a number of gels (more than 100 gels visible on screen at once). The user chooses the various options with pop-up menus organized so that most operations involved in common tasks appear on the same menu. Any part of any gel in the experiment can be inspected in a multi-gel context. Gels can be brought into register interactively so that identical regions can be compared.

AUTOMATIC MATCHING. Beginning with a few spot matches entered interactively, the system compares sets of patterns and establishes all reliable matches to the master pattern. Parameter files supplied with the software control the emphasis placed on positional, shape, and intensity similarities required for declaring matches. These parameter sets are chosen by the user based on the type of experiment being analyzed (whether few or many changes are expected, etc.).

GEL RESOLUTION MEASUREMENT. A measure of gel resolution is computed based on the optimized spot model (**40**). This measure is recommended for use in monitoring gel quality for QC purposes.

10.6 Databases

A major motivation behind the development of 2-D technology has been the desire to build comprehensive databases describing the proteins present in living systems (**41**,**42**,**43**). Given a computer system capable of

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40) Numerical measures of two-dimensional gel resolution and positional reproducibility. Taylor, John, Anderson, N. Leigh, and Anderson, Norman G., Electrophoresis 4: 338-345, 1983.

41) Molecular anatomy. Anderson, N.G. and Anderson, N.L., Behring. Inst. Mitt. 63: 169-210, 1979.

42) The human protein index. Anderson, Norman G. and Anderson, Leigh, Clin. Chem. 28: 739-748, 1982.

43) Electrophoresis and large-scale databases. Anderson, Norman G., Science 235: G65, 1987.

analyzing the gels, the obvious next step is to organize and preserve the data in a database. Despite the familiarity of the term "database" and the glibness with which it has come to be used, building a database is not a trivial undertaking. Constructing a generally-useful database requires a level of effort comparable to writing a book (recognizing that there are small books, large books and encyclopedias: much depends on the scope of the research being done). The approaches required are thus somewhat beyond the scope of this manual. Nevertheless, several interesting principles have emerged from the efforts of those beginning to build and use such databases, and these deserve mention.

First, it should be noted that the quality of a database is only as good as the gels from which the data is obtained. Gel quality includes both resolution and matching accuracy (the reliability with which spots can be identified through a series of gels). Before investing the effort involved in codifying a project in database form, it is worthwhile to tune your gels to perfection. This is not only the most rigorous approach - it is also the most economical. Good gels are much easier to analyze and to match than marginal gels because the computer algorithms yield a very much higher proportion of accurate data from them. Much less inspection, editing and "hand" matching work at the computer is required to produce a reliable data set.

Second, there is the matter of database type. A least two quite different kinds of database can be envisioned in this field, and it is important to have a clear idea of whether you want one or the other (or both). On the one hand, there is a database constructed primarily of numerical quantitative data drawn from measurements off gels, a database most appropriate for answering questions about the nature of statistically significant differences between samples. We have called this a "quantitative" or "effects" database. On the other hand, there is a database containing information, often drawn from outside sources, describing spots, sets of spots, etc.. We call this an "annotational" database, since it is comprised mainly of textual information. In general, it appears that most users will generate effects-type databases through work in a specific experimental system, draw conclusions that focus attention on one or more proteins, and then turn to annotational databases to provide all the available characterization of these molecules.

Third, there is the issue of connecting databases. Clearly the best way to extract information from a 2-D database is through the protein pattern itself, i.e., by running gels that match the database gels. It is possible, generally by brute force methods, to identify corresponding proteins on non-identical 2-D maps. This will ultimately make it possible to link together different 2-D databases. However, this has yet to be demonstrated as a general approach, and, once again, it is best to agree with collaborators on a single gel system and database architecture.

Lastly, it should be pointed out that a good start can be made on an annotational database without a computer system (provided you are running reproducible gels). Starting with an enlarged print of a "standard" gel of the sample in question, a lot can be done just by keeping notes on the pattern itself: writing down spot identifications, characteristics, major qualitative changes, hints, suspicions, etc.. This information is directly publishable (as a figure), and can later be incorporated as text into an annotational computer database. The figures that follow represent such "standard maps" used at LSB as foundations for the development of computer databases. LSB is building databases in several areas including plasma proteins (see Figures in section 11.2), human lymphocytes (see Figure 10.6.1), the mouse and rat (particularly liver; see Figure 10.6.2), muscle proteins (Figures 10.6.4 & 10.6.5) and various plants (Figures 10.6.6 & 10.6.7).

Figure 10.6.1: The following page shows an autoradiograph of a 7"x7" ISO-DALT pattern of human mononuclear leukocytes labeled with ^{35}S methionine and showing a number of identified proteins (**44**).

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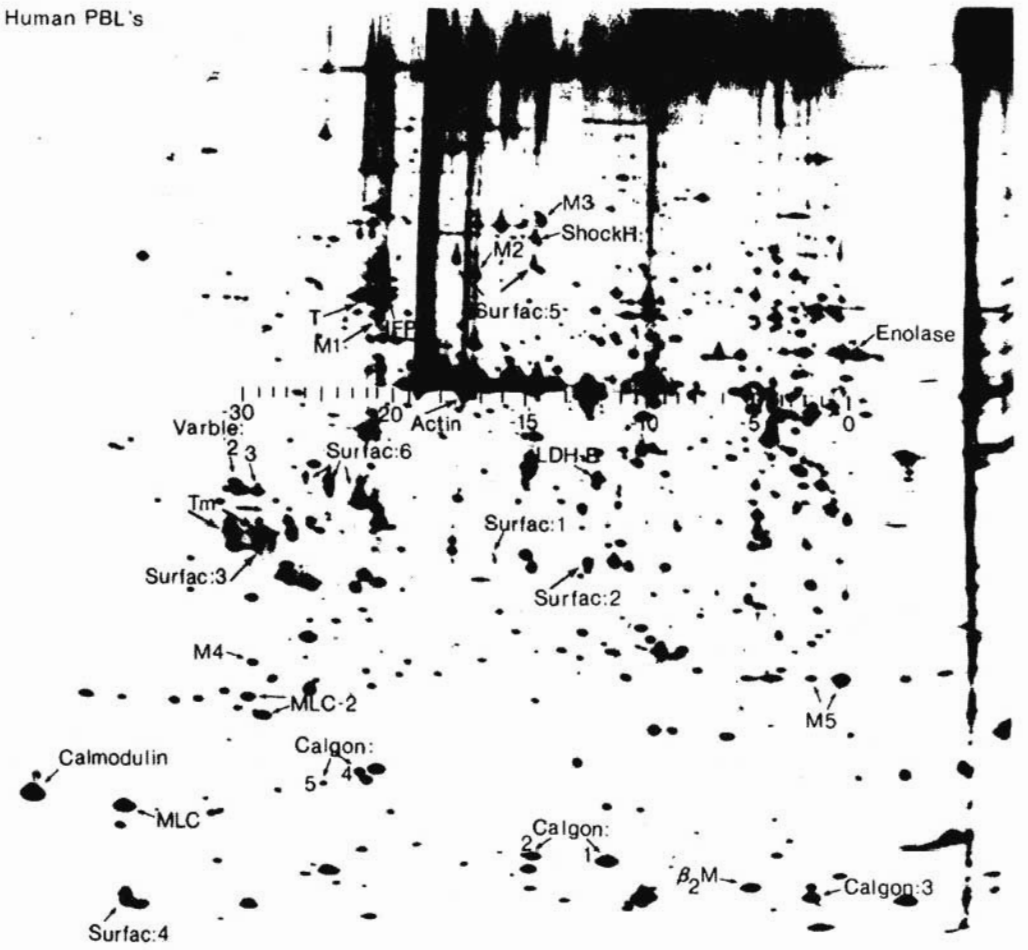
44) Reprinted with permission from the AACC from Clinical Chemistry (1981) Vol. 27, No. 11, (The TYCHO system for computer analysis of two-dimensional gel electrophoresis patterns. Anderson, N. L., Taylor, J., Scandora, A. E., Coulter, B.P., and Anderson, N. G.), Figure 8. Copyright American Association for Clinical Chemistry, Inc.



Autoradiograph of a separation of human peripheral blood leukocyte proteins labeled with 35S-methionine

Several identified proteins are labeled including actin, tubulin (*T*), lymphocyte intermediate filament protein (*IFP*), β_2 microglobulin (β_2-*M*), calmodulin, enolase, lymphocyte tropomyosins (Tm), the LDH-B chain, and putative myosin light chains (*MLC*). Members of some prominent protein sets are also indicated, with their numbers in the set: surface proteins (*Surfac*), mitochondrial proteins (*Mitcon*, labeled M), heat shock protein (*ShockH*), calcium-regulated proteins (*Calgon*), and variable proteins (*Varble;* control unknown). The scale running horizontally at the level of actin shows the positions of creatine kinase charge standards

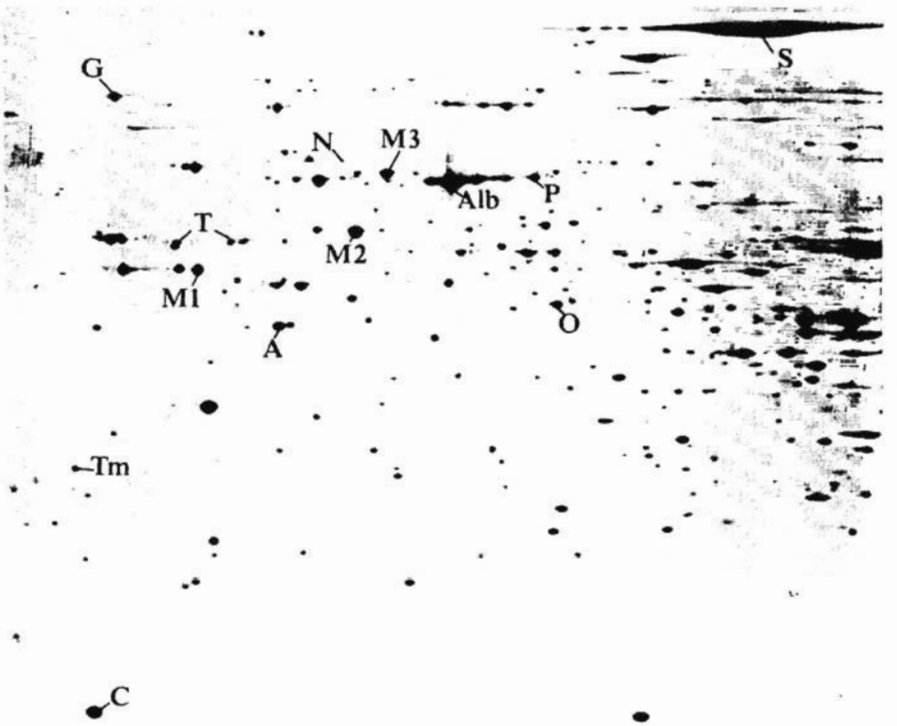


Figure 10.6.2: Above: an 8"x10" Coomassie Blue-stained ISO-DALT pattern of mouse liver showing identified proteins. M1, M2 and M3 are major mitochondrial proteins (M1 is the β subunit of the F_1 ATPase), A is actin, Alb is serum albumin, P is the albumin precursor polypeptide, C is cytochrome b_5, N is the NADPH cytochrome c reductase, S is carbamyl phosphate synthetase, T is tubulin, O is ornithine aminotransferase and G is a major heat-shock-inducible glycoprotein of the Golgi apparatus.
Figure 10.6.3: On the following page: Coomassie Blue-stained patterns identifying subcellular location of proteins in mouse liver: (A) total protein, (B) mitochondria [circles], (C) microsomes [squares] and (D) soluble proteins [triangles]. Reproduced from **45**.

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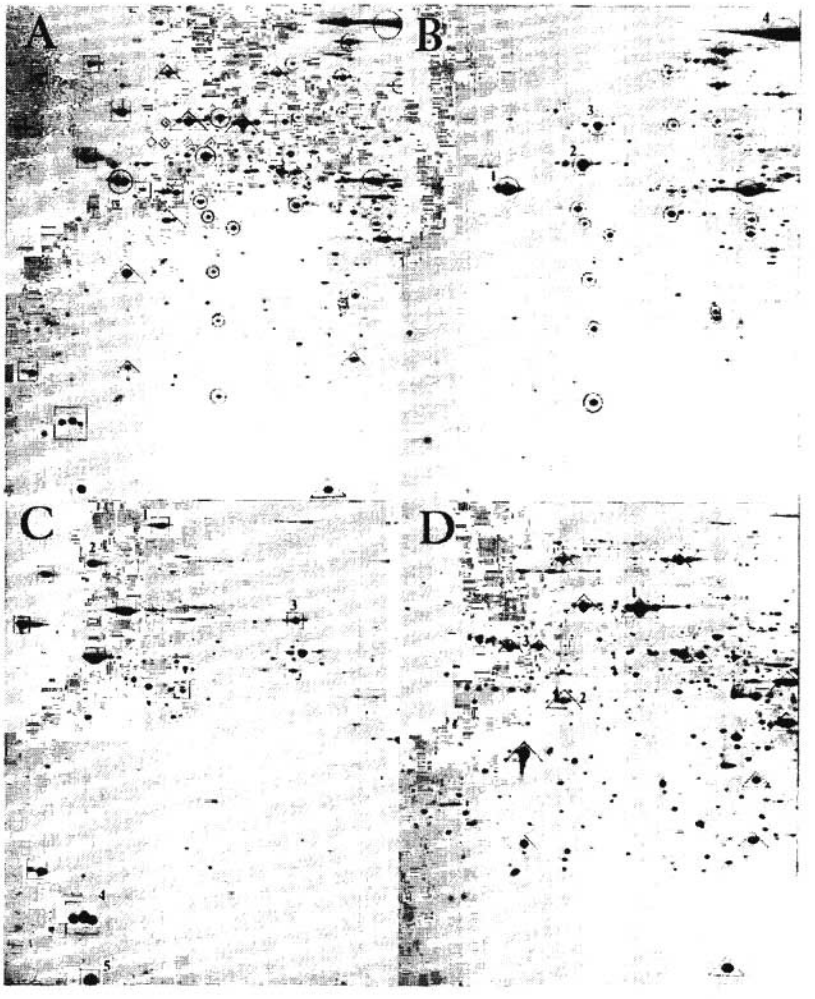
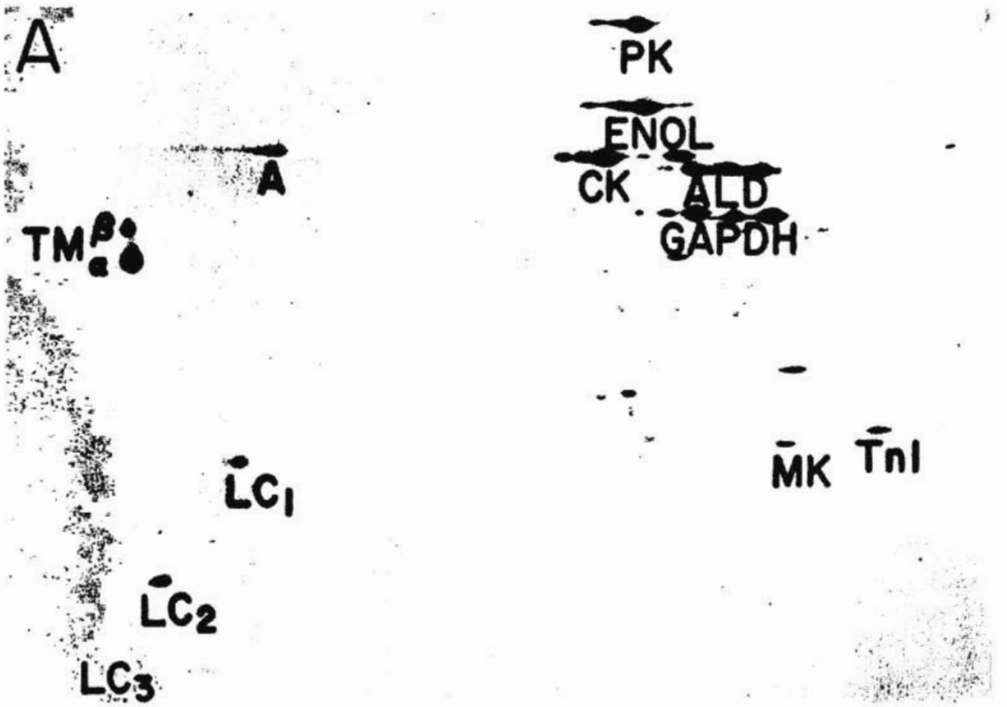
45) Effects of toxic agents at the protein level: Quantitative measurement of 213 mouse liver proteins following xenobiotic treatment. Anderson, N. Leigh, Giere, Frederic A., Nance, Sharron L., Gemmell, M. Anne, Tollaksen, Sandra L., and Anderson, Norman G., Fundamental and Applied Toxicology 8: 39-50, 1987.



Two-dimensional protein patterns of unfractionated liver (A), a mitochondrial fraction (B), a microsomal fraction (C), and the soluble phase (D). Major proteins of each fraction are indicated in the unfractionated pattern in (A) and in the respective fraction pattern: mitochondrial proteins are enclosed in circles, microsomal proteins in squares, and some soluble proteins in triangles. Nuclear proteins, which represent only minor components in this pH range, are enclosed in diamonds. Circles labeled 1–3 are the proteins designated Mitcon:1–3 in previous studies of human cells (Anderson, 1981). Mitcon:1 is the β subunit of the F1 ATPase, and is hence part of the inner membrane. Mitcon:2 and :3 are mitochondrial matrix polypeptides (Anderson, 1985). Circle 4 is likely to be carbamyl phosphate synthetase by analogy with the rat mitochondrial protein pattern (Henslee and Srere, 1979). Squares 1 and 2 are known by analogy with studies of human proteins to be glycoproteins not present on the cell surface (and hence likely to reside in internal membranes). Square 2 is likely to be a major Golgi apparatus protein (Lin *et al.*, 1982). Square 3 is likely to be the proalbumin polypeptide, and square 5 is almost certainly cytochrome b_5. Square 4 is a protein expressed predominantly in males though at quite variable abundance. Triangle 1 is serum albumin, triangle 2 is actin, and triangles 3 are β and α tubulin (left to right).



Two-dimensional patterns of rabbit psoas muscle whole homogenate and myofibrils, with nonequilibrium pH gradient electrophoresis in the first dimension

Figure 10.6.4: Protein pattern of rabbit muscle, and identification of major polypeptides. Reproduced from **46**.

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46) Reprinted with permission from the AACC from Clinical Chemistry (1979) Vol. 25, pp 1877-1884 (Muscle Protein Analysis. I. High resolution two-dimensional electrophoresis of skeletal muscle proteins for analysis of small biopsy samples. Giometti, Carol Smith, Anderson, Norman G., and Anderson, N. Leigh) Copyright American Association for Clinical Chemistry, Inc.

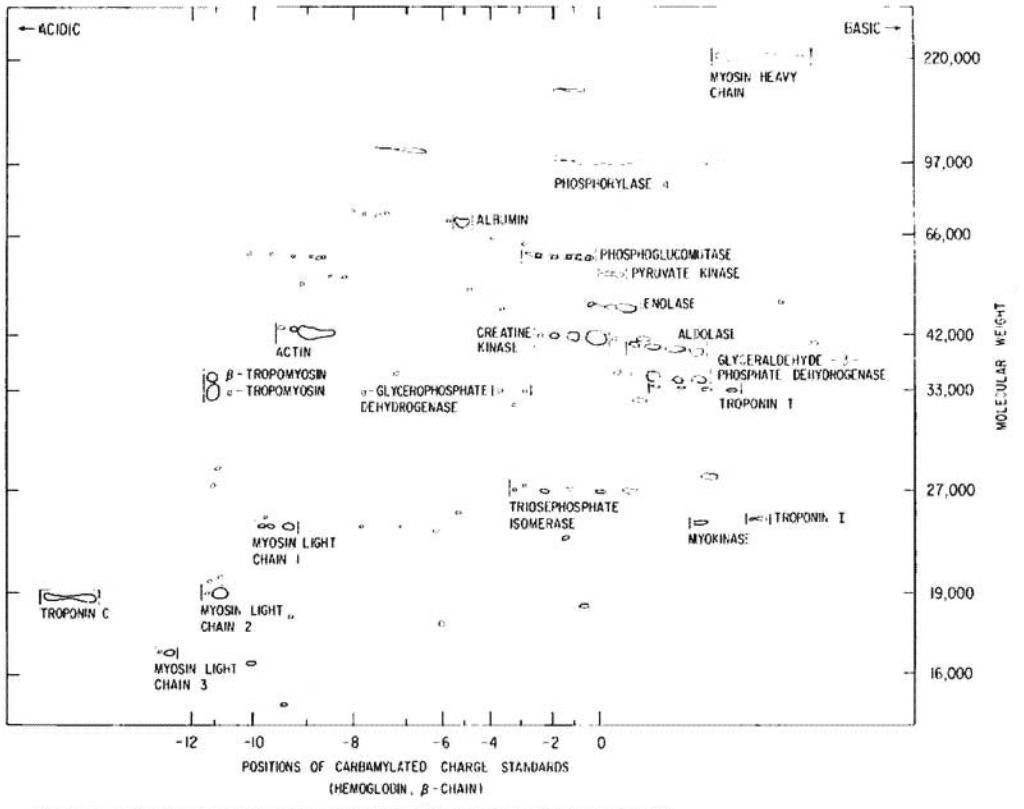


Diagram of the two-dimensional pattern of rabbit muscle whole homogenate

Protein identifications were made by co-electrophoresis of purified muscle proteins. Molecular weight calibrations (*ordinate*) obtained with rat heart strenk standards ; positions of carbamylated charge standards (*abscissa*) determined with carbamylated hemoglobin

Figure 10.6.5: Diagram of rabbit muscle pattern, with identification of major polypeptides (derived from Figure 10.6.4 and reproduced from the same source).

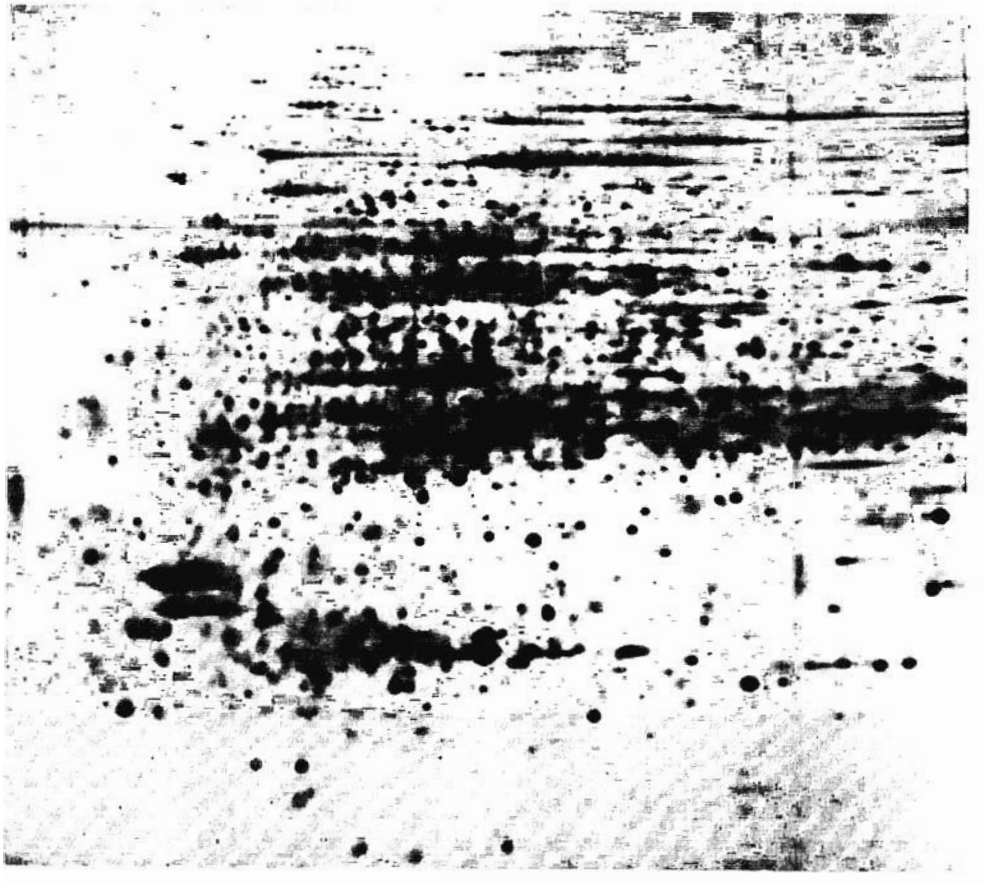
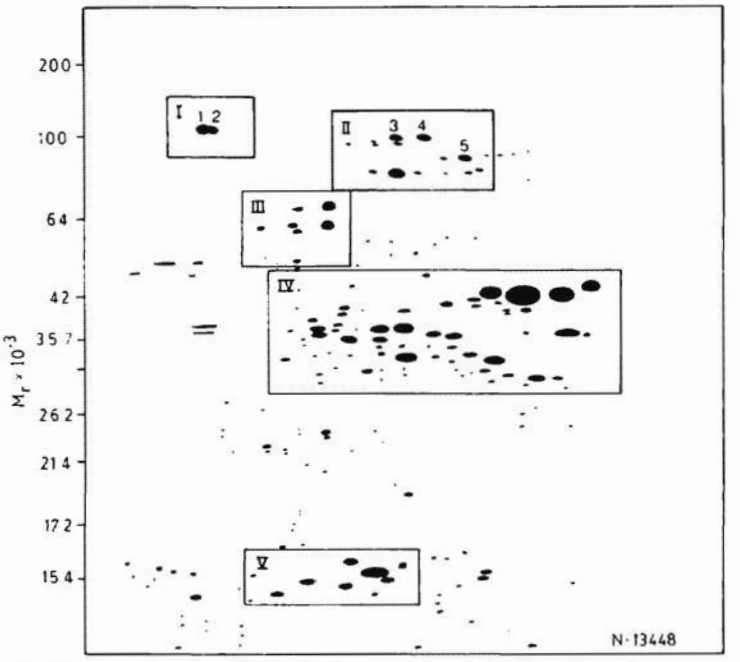


Figure 10.6.6: Silver-stained 2-D pattern of proteins from a corn shoot.



Reference map for Chinese Spring wheat with molecular mass values derived from internal mass standards shown to the left.

Figure 10.6.7: Diagram derived from a Coomassie Blue-stained 2-D pattern of proteins from a grain of wheat (reproduced from **47**).

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47) Two-dimensional electrophoretic analysis of wheat seed proteins. Anderson, Norman G., Tollaksen, Sandra L., Pascoe, Frank H., and Anderson, Leigh, Crop Science 25: 667-674, 1985, by permission of the Crop Science Society of America, Inc.

11 IEF and SDS Positional Standardization

Positional standardization is vital for systematic use of 2-D gels. In the course of developing the ISO-DALT® system, standardization systems have been developed for both dimensions. The standards are useful both for quality control and for indicating positions of protein spots of interest.

11.1 IEF Standardization Using CPK Carbamylation Trains

By taking a rather basic protein and progressively modifying more and more of its lysine amino groups (by carbamylation), a series of molecular forms can be generated that span a broad range of isoelectric points in discrete steps (**48,49,50**). Using rabbit muscle creatine phosphokinase (CPK; easily obtained from Sigma), a series of about 30 charge isomers is generated (see recipe section) that span almost the whole pI range obtained with typical wide-range ampholytes. Such a standard mix is now also commercially available from Pharmacia (Carbamylytes™) and from BDH (as a 2-D pI calibration kit, range 4.95-7.0).

Position in the train is counted negatively, starting with the unmodified molecule as zero. This gives a position scale that runs in the same sense as the pH scale and the Cartesian coordinate system: from low values on the left to high values on the right. The position value also corresponds to the net change in the charge of the modified protein: at position -5, five net positive charges have been removed.

Positions of interesting proteins can be specified accurately in this system because the standards are internal to the separation, i.e., they are run in the same gel as the sample. Cellular β actin can thus be identified as the protein with a mass almost the same as the standards and a position of -17.2

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48) Analytical techniques for cell fractions. XXIV. Isoelectric point standards for two-dimensional electrophoresis. Anderson, N.L. and Hickman, B.J., Anal. Biochem. 93: 312-320, 1979.

49) Internal charge standardization for two-dimensional electrophoresis. Hickman, B.J., Anderson., N.L., Willard, K.E., and Anderson, N.G., Electrophoresis '79, B. Radola, ed., W. de Gruyter, Berlin, pp 341-350, 1980.

50) The use of carbamylated charge standards for testing batches of ampholytes used in two-dimensional electrophoresis. Tollaksen, Sandra L., Edwards, Jesse J., and Anderson, Norman G., Electrophoresis 2: 155-160, 1981.

relative to the CPK train. CPK standards can be used to reveal the smoothness (or lack of it) of IEF pH gradients generated by commercial preparations of ampholytes (see figure in Iso chapter).

11.2 SDS Standardization Using the Human Plasma Proteins

By applying a small amount of human serum, plasma or whole blood (2.5 µl of a 1:3 dilution in solubilization mix) along with a representative sample on a single gel, a combined pattern is produced that allows good SDS molecular mass standardization of the sample. The plasma 2-D pattern contains more than 40 identified proteins, a number of which are easy to find in any good 2-D pattern (see Figures 11.2.1, 11.2.3). By locating these proteins in the combined pattern, a standard curve of mobility versus molecular mass can be created (**51**).

The plasma pattern is generally quite consistent from lab to lab and thus also represents one of the best samples with which to "tune-up" a 2-D operation.

A list of some plasma proteins that are easy to find in the pattern and have well-established molecular masses (**52**) includes:

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51) High-resolution two-dimensional electrophoretic mapping of plasma proteins. Anderson, N. Leigh, Tracy, Russell P., and Anderson, Norman G., In: The Plasma Proteins, F. Putnam, ed., Academic Press, 2nd Ed., Vol. 4, pp 221-270, 1984.

52) The Plasma Proteins, F. Putnam, ed., Academic Press, 2nd Ed., Vol. 4, pp 48-61, 1984.

Plasma Protein	Mass
α_2 Macroglobulin	181,250
Ceruloplasmin (higher MW form)	132,000
Transferrin	79,550
Albumin	66,458
Hemopexin	60,000
α_1 Antitrypsin	54,000
Apo A4 (≅MW actin)	42,000
Haptoglobin β	33,820
Apo A-I	28,076
Haptoglobin α_2	15,939
Transthyretin (Prealbumin)	13,745
Hemoglobin β	15,500
Haptoglobin α_1	9,189
Apo C-III	8,764
Apo A-II	8,707

The following figures show the plasma 2-D pattern, the derived standard maps and a standard curve relating mobility to mass for a 8-18%T gradient ISO-DALT gel.

Two-dimensional gel of human plasma proteins. The sample was 10 µl of fresh heparinized plasma denatured in Na-$DodSO_4$/mercaptoethanol.

Figure 11.2.1: Coomassie-Blue stained gel of fresh heparinized human plasma solubilized using SDS mix and run on 7"x7" ISO-DALT gels. Reproduced from (**53**).

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53) High resolution two-dimensional electrophoresis of human plasma proteins. Anderson, Leigh and Anderson, Norman G., Proc. Nat. Acad. Sci. USA 74: 5421-5425, 1977.

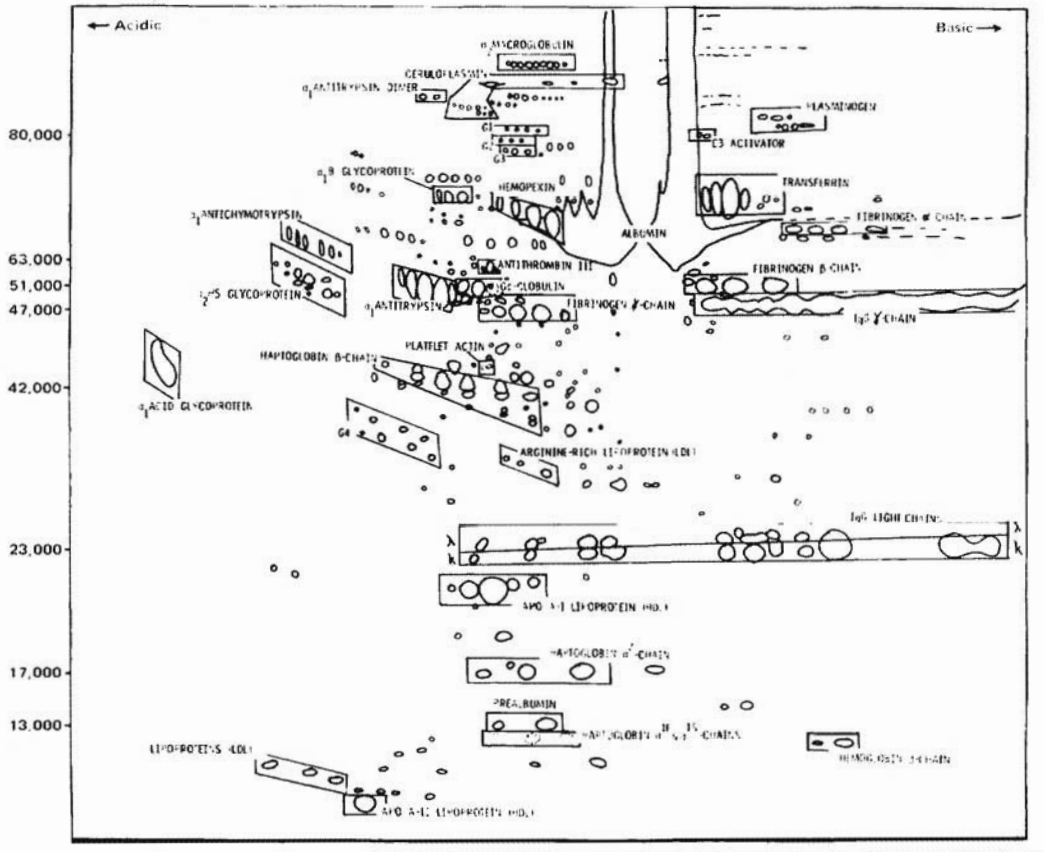

Diagram drawn from the gel shown and labeled to indicate positions of known plasma proteins. Hemopexin and the C3-activator are somewhat obscured by albumin overloading. Ceruloplasmin appears to be present in two major and two minor forms (all between 80,000 and 90,000 daltons), each present as a row of four or more dots due to sialic acid heterogeneity. The highest molecular weight form interacts strongly with the albumin precipitate, while the others do not. Plasminogen exists in two forms: the Glu-form (upper horizontal row of dots) and the Lys-form (lower row, more basic) . Gc-globulin can be present as three spots; the left-hand pair appears to correspond to type 1, and the right-hand spot to the type 2 allele. The immunoglobulin light chains (κ and λ) are partially resolved (2D) and show similar isoelectric distributions. Identification of the lipoproteins is based on the presence of spots in certain of the low (LDL) and high (HDL) density lipoprotein fractions, as well as similarity to isolated materials for the arginine-rich and apo A-I lipoproteins. Platelet actin, Gc-globulin spot 3, and the haptoglobin α^{1F} and α^{1S} chains are shown although they were not present in the sample run in Fig. . As yet unrecognized glycoproteins G1, 2, 3, and 4 are labeled for use in the *text*. The hemoglobin α-chain is too basic to appear in a separation with these ampholytes.

Figure 11.2.2: The original map of human plasma, drawn from the pattern in Figure 11.2.1 (reproduced from the same source).

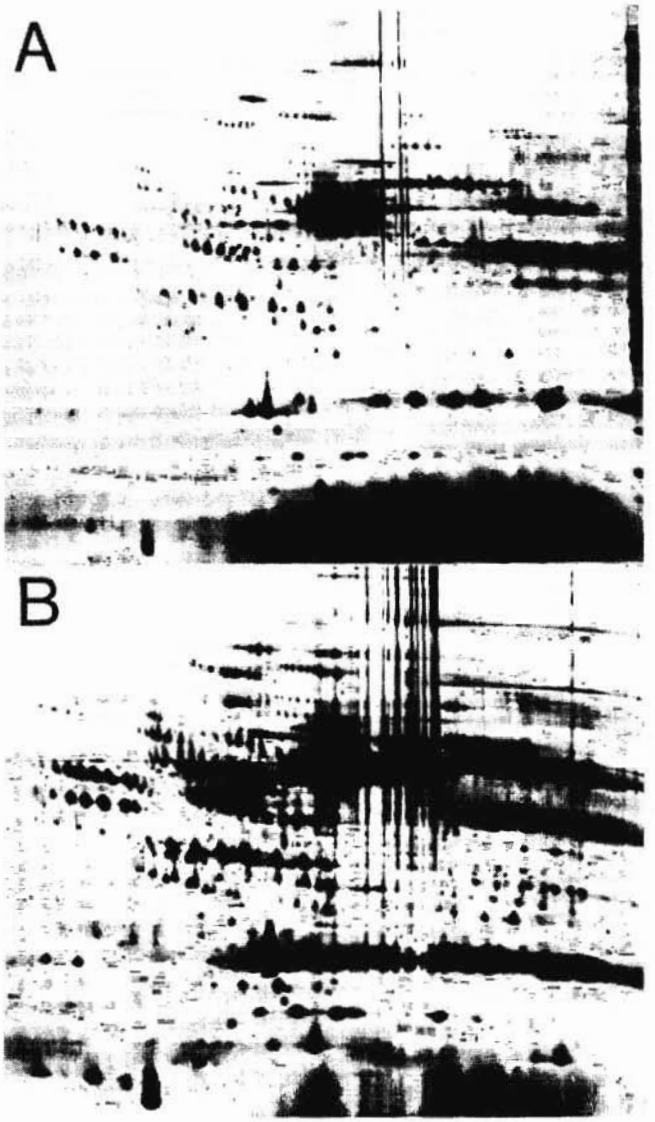

Figure 11.2.3: Silver-stained human plasma protein patterns at 1 µl (A) and 10 µl (B) loadings on 8"x10" ISO-DALT gels (reproduced from **54**).

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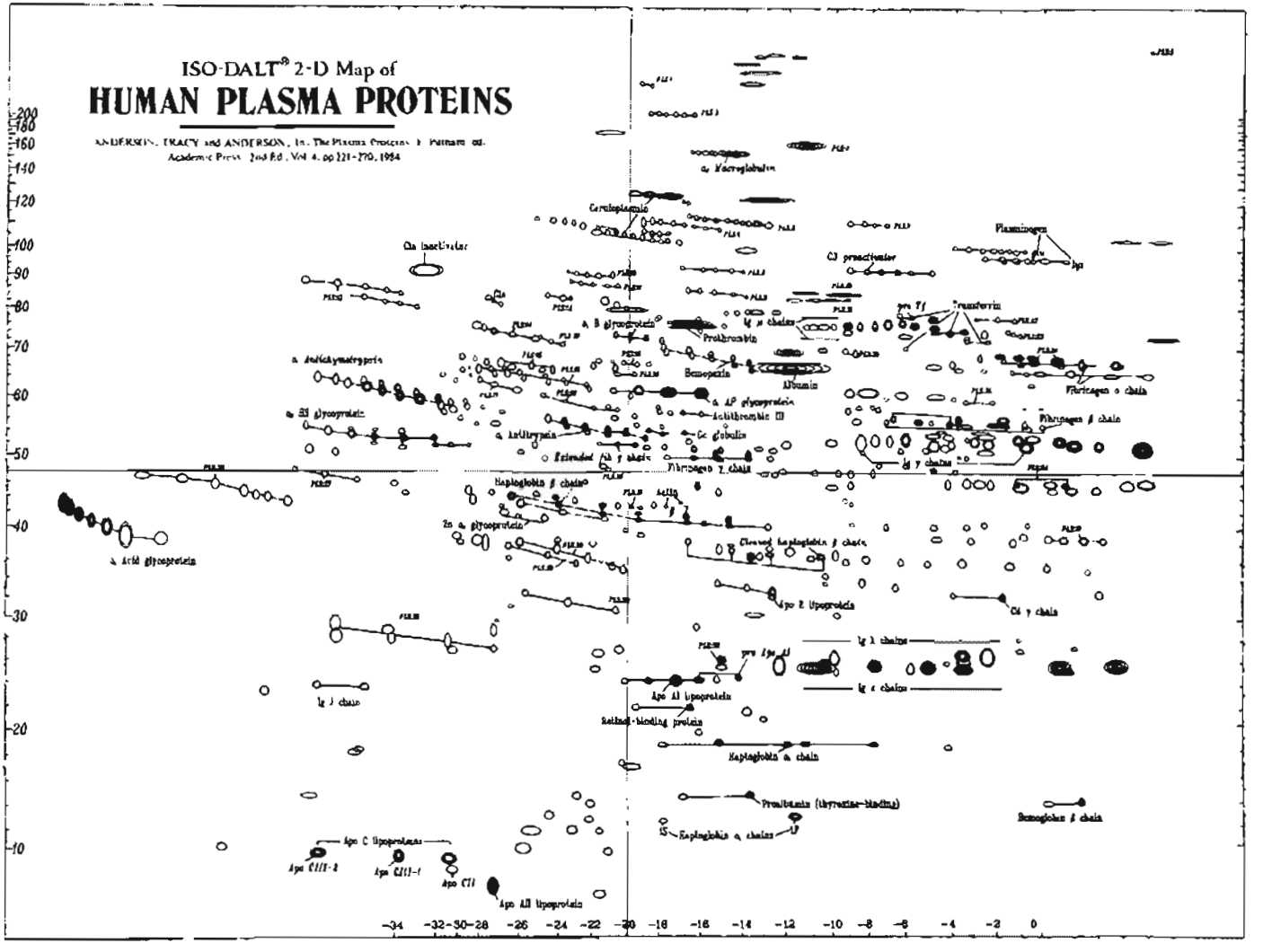
54) High-resolution two-dimensional electrophoretic mapping of plasma proteins. Anderson, N. Leigh, Tracy, Russell P., and Anderson, Norman G.. In: The Plasma Proteins, F. Putnam, ed., Academic Press, 2nd Ed., Vol. 4, pp 221-270, 1984.

Figure 11.2.4: On the next page: Standard human plasma protein map constructed via computer analysis from the gels shown in Figure 11.2.3 (reproduced from the same source).


ISO-DALT® 2-D Map of
HUMAN PLASMA PROTEINS
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α2 Macroglobulin
Ceruloplasmin
C1 inactivator
C1 proactivator
Plasminogen
Transferrin
α1 B glycoprotein
Prothrombin
Hemopexin
Albumin
α1 Antichymotrypsin
α1 AP glycoprotein
α1 Antitrypsin
Inter-α-trypsin inhibitor
Fibrinogen α chain
Fibrinogen β chain
Fibrinogen γ chain
Haptoglobin β chain
Zn α2 glycoprotein
α1 Acid glycoprotein
Cleaved haptoglobin β chain
Apo E lipoprotein
Cλ γ chain
Ig λ chains
Ig κ chains
Ig λ chain
Apo AI lipoprotein
Retinol-binding protein
Haptoglobin α chain
Prealbumin (thyroxine-binding)
Haptoglobin α chains
Hemoglobin β chain
Apo C lipoproteins
Apo CIII-2
Apo CIII-1
Apo CII
Apo AII lipoprotein
200
180
160
140
120
100
90
80
70
60
50
40
30
20
10
-34 -32 -30 -28 -26 -24 -22 -20 -18 -16 -14 -12 -10 -8 -6 -4 -2 0

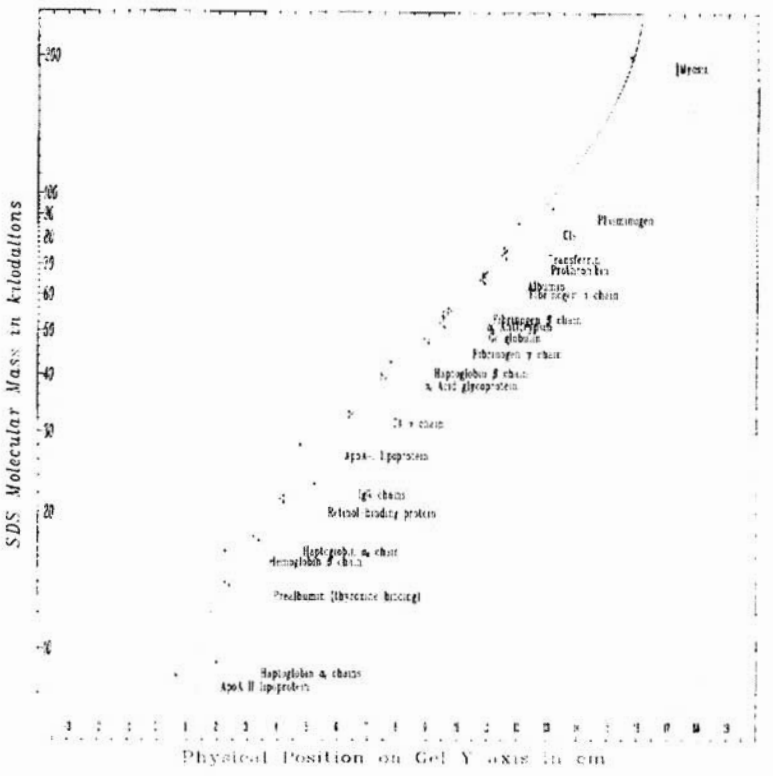

Figure 11.2.5: SDS standardization curve for 8-18%T ISO-DALT slab gels (**55**). The figure illustrates the use of numerous standards (in this case, plasma proteins) to calibrate the SDS separation. Here, using a linear gradient gel, the standard curve is best fit by a fourth-order polynomial. While relative masses are very accurately determined (±1%), absolute masses are only accurate to ±5-10% in the SDS system due to variations in the SDS-binding stoichiometries of different proteins.

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12 Reagents

Reagent	MW	Comments
Acrylamide	71.1	Very high purity required for Iso dimension; may use less pure for Dalt (except when micro-sequencing). Reported to be a neurotoxin: HANDLE with CARE.
Agarose		Purified.
Ammonium persulfate	228.2	The solid can deteriorate in bottle. Refrigerate.
Bis (N,N'-methylene- bisacrylamide)	154.2	High purity. HANDLE with CARE.
Bromophenol Blue	692.0	Tracking dye used in trace quantities.
CHAPS	614.9	Zwitterionic detergent sometimes used as an alternative to NP-40.
CHES		pK_a 9.3 buffer used to raise pH of some samples.
Coomassie Brilliant Blue R250	849.	Some commercial grades contain substantial filler: dissolve in alcohol, and filter.
DTT (dithiothreitol)	154.2	Smells less than mercaptoethanol.
Glycerol	92.1	Reagent
Glycine	75.1	Reagent
Mercaptoethanol	78.3	Reagent
NP-40 (Nonidet P-40)		Sigma. Store cold in the dark. Density: 1.06.
Phosphoric acid	98.	Reagent
SDS (sodium dodecyl sulfate)	288.4	Lot-to-lot variation is a problem: try to obtain large batch. Major feature is % of C_{12} isomer: should be at least 98-99%.
Sodium hydroxide	40.	Reagent
TEMED (tetramethyl ethylenediamine)	116.2	Avoid inhalation.
Tris base	121.1	Pure grade.
Tris HCl	157.6	Pure grade.
Urea	60.1	Very pure grade required.

13 Solutions Used in the ISO-DALT® System

13.1 Iso Gel Mix

Batch size: 16 ml
pH depends on ampholytes; need not be measured
Density: (@ 21°C)
Storage: make fresh; not to be stored

Reagent	Concentration	Density (g/ml)	Weight (g)	Volume (ml)
Urea	9 M		8.74	
Acrylamide Stock (recipe 13.2)	3.30 %T	1.021	1.715	1.68
NP-40 Stock (20% in water)	2 %	0.951	1.540	1.62
Ampholytes	2 %	1.144	0.915	0.80
Water		0.990	5.116	5.17
Ammonium Persulfate (10%)				0.100
TEMED (10%)				0.100

Directions: Mix the first five components, taking care to dissolve the urea. Degas, if desired. Add TEMED and Ammonium Persulfate, and rapidly cast the gels.

13.2 Iso Acrylamide Stock (31.8%T)

Batch size: 50 ml
pH NA
Density: 1.026 g/ml (@ 21°C)
Storage: 4°C for ≤ 1 month

Reagent	Concentration	Weight (g)	Volume (ml / g)
Acrylamide (purest grade)	30%	15	
Bis (N,N' methylenebis-acrylamide, purest grade)	1.8%	0.9	
Water (purest available)		~35.35	to 50 ml (~51.25 g)

Directions: Weigh acrylamide and bis under a hood to avoid contact with dust. Filter and store refrigerated. Never mouth pipette acrylamide solutions and wear gloves when handling them. Acrylamide is a NEUROTOXIN.

13.3 Dalt Casting Recipes Using Gravity Gradient Maker

13.3.1 Dalt Acrylamide Stock (30.8%T)

Batch size: 3,000 ml
pH NA
Storage: 4°C

Reagent	Concentration	Weight (g)	Volume (ml)
Acrylamide (best affordable grade)	30%	900	
Bis (N,N' methylenebis- acrylamide, purest grade)	0.8%	24	
Water (purest available)			to 3,000

Directions: May need filtration. Weigh acrylamide and bis under a hood to avoid contact with dust. Filter and store refrigerated. Never mouth pipette acrylamide solutions and wear gloves when handling them. Acrylamide is a NEUROTOXIN.

13.3.2 Buffer "II" (2x buffer for optional stacking gels)

Batch size: 375 ml
pH 6.8 (using HCl)
Storage: 4°C

Reagent	Concen-tration	Weight (g)	Volume (ml)
Tris base	0.25 M	11.35	
SDS	0.2 %	0.75	
HCl (6N in water) to pH 6.8			
Water			to 375 total

13.3.3 Buffer "D" (4x buffer for Dalt slab gels)

Batch size: 3,000 ml
pH 8.6
Storage: 4°C

Reagent	Concen-tration	Weight (g)	Volume (ml)
Tris base	1.5M	545	
6N HCl to pH 8.6			about 150
Water			to 3,000

13.3.4 10% Ammonium Persulfate

Batch size: 50 ml
pH NA
Storage: 4°C in a dark bottle

Reagent	Concen-tration	Weight (g)	Volume (ml)
Ammonium persulfate	10%	5	
Water	90%		to 50

13.4 Dalt Casting Recipes Using Angelique™

See Angelique chapter, section 7.14.

13.5 Iso Equilibration Buffer

Batch size: 750 ml
pH 6.8
Density: 1.026 g/ml (@ 21 °C)
Storage: 4°C

Reagent	Concentration	Weight (g)	Volume (ml)
Glycerol	10 %		75
Dithiothreitol (DTT)	4.9 mM	1	
SDS	2 %	15	
Bromophenol Blue	trace		
Tris base	0.125 M	11.35	
HCl (6N in water)			to pH 6.8
Water			to 750 total

Directions: An easier variant of this recipe is replacing Tris base and HCl for Tris Premix 6.8. The same amount required for Tris base in the above recipe is valid for Tris Premix in the modified one.

13.6 Running Buffer Agarose for Sealing Iso in Place on Dalt

Batch size: 1,000 ml
pH 8.6
Storage: frozen (-20°C) in aliquots

Reagent	Concentration	Weight (g)	Volume (ml)
Tris base	24mM	2.9	
Glycine	0.2M	15.0	
SDS	0.1%	1.0	
Agarose	0.5%	5.0	
Water			to 1,000

Directions: Boil to dissolve, aliquot and freeze.

13.7 Dalt Tank Electrophoresis Buffer

Batch size: see table below
pH 8.6
Density: 1.002 g/ml (@ 21°C)
Storage: make fresh by dissolving in tank

Gels/Tank	20	20	10	10	4	4
Gel Size	7"x7"	8"x10"	7"x7"	8"x10"	7"x7"	8"x10"
Vol. (l)	29.7	38.2	15.5	19.9	6.9	9.0
Tris base (g) (24mM)	86.2	111.0	44.9	58.0	20.2	26.2
Glycine (g) (0.2M)	445.6	573.6	232.3	299.6	104.3	135.2
SDS (g) (0.1%)	29.7	38.2	15.5	19.9	6.9	9.0

Directions: Solid reagents can be added directly to the central chamber of the tank once it is filled with the appropriate volume of pure water. The tank buffer circulator can then be used to dissolve the buffer components; this takes about 1-2 hours depending on water temperature. Remember to fill the tank initially using water near room temperature, and to raise the two seal combs to allow mixing of central and electrode chambers before loading cassettes and starting the run.

Dalt tank buffers, though made in large volumes, do not constitute a large fraction of the cost of running gels: a fill for 20 gels costs about $13. The major feature of the Dalt approach is the tank's ability to "make up" the buffer from a pre-weighed packet without using other vessels.

13.8 Fix Stain Solution

Batch size: 20,000 ml
pH
Density: 0.931 g/ml (@ 21°C)
Storage: at room temperature

Reagent	Concentration	Weight (g)	Volume (ml)
Alcohol (ethyl)	50%		10,000
Phosphoric acid 85%	2%		200
Water	48%		9,800

13.9 Neuhoff Concentrate

Batch size: 20,000 ml
pH
Density: 1.058 g/ml (@ 21°C)
Storage: At room temperature.

Reagent	Concentration	Weight (g)	Volume (ml)
Alcohol (methyl)	34%		6,800
Phosphoric acid 85%	3%		600
Ammonium Sulfate	17%	3,400	
Water	46%		12,000

13.10 Standard Urea/NP-40 Sample Solubilizer: Pink Mix

Batch size: 100 ml
pH >9.5
Storage: frozen -70°C in 1 ml aliquots

Reagent	Concentration	Weight (g)	Volume (ml)
Urea	9M	54	
NP-40 detergent	4%		4
Ampholyte (8-10 or 9-11 range; 20% stock)	2%		10
DTT (dithiothreitol)	1%	1	
Water			to 100

Directions: Adjust pH with minimum NaOH if necessary.

13.11 Urea/NP-40 Sample Solubilizer for BASO Samples

Batch size: 20 ml
pH 3.0
Storage: frozen -70°C in 1 ml aliquots

Reagent	Concentration	Weight (g)	Volume (ml)
Urea	9M	10.8	
NP-40 detergent	4%		0.8
Ampholyte (Serva 3-10; 40% stock)	2%		1.0
DTT	1%	0.2	
Water			to 20.0

Directions: Adjust pH with minimum H_3PO_4 if necessary.

13.12 SDS Sample Solubilizer

Batch size: 10 ml
pH 9.5
Storage: frozen -70°C in 1 ml aliquots

Reagent	Concen-tration	Weight (g)	Volume (ml)
CHES	0.05M	0.1	
SDS	2%	0.2	
DTT	1%	0.1	
Glycerol	10%		1
Water			to 10

13.13 UKS Extraction Mixture for Plant Samples

Batch size: 80 ml
pH
Storage: frozen -70°C

Reagent	Concen-tration	Weight (g)	Volume (ml)
Urea	9.5M	45.6	
K_2CO_3 (5M in water)	0.125M		2.0
DTT	0.03M	0.4	
SDS	1.25%	1.0	
Ampholytes (LKB 3.5-9.5; 40%)	2.0%		4.0
Water			34.0

13.14 Preparation of Creatine Kinase (CK) Charge Standards

- Dissolve 5 mg of rabbit muscle creatine phosphokinase (Sigma) in 1 ml of a solution of 8M urea and 1% mercaptoethanol, to give a concentration of 5 mg CK/ml.

- Aliquot the above mixture into 7 tubes.

- Do not heat the first tube. Heat each of the remaining 6 tubes for 4, 6, 8, 10, 12, and 15 minutes at 95°C in a heating block (or in a boiling water bath). At the end of each time period, place the appropriate tube in an ice bucket.

- The 7 tubes are then mixed together, and aliquots of 50 µl of the pool are apportioned into small microfuge tubes for storage at -70°C.

- Thaw out a tube for each experiment and load 2 µl CK mix on top of each Iso tube containing the protein sample to be run.

14 User's Notes

User's Notes

15 LSB References

The following is a list of publications by LSB staff in the areas of 2-D electrophoresis technology and its applications. These papers all represent work related to the ISO-DALT® system and include basic 2-D maps from a wide range of experimental systems.

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16 ISO-DALT® Run Sheet

The following page shows a prototype run sheet for recording the most useful information during the course of both Iso and Dalt runs. You can copy this page onto full-size (8 1/2" x 11") paper using a copier that will enlarge to 125%. It is convenient to set up a looseleaf notebook of such sheets, one for each run. This provides a central register of all gels (and all samples) run.

ISO-DALT® Run Sheet

Experiment No. _____

Protocol:
Title of Experiment:
Date:

Initials:
Iso Unit:
Dalt Tank:

Dalt Gel No.	Iso No.	Sample Vol.(µl)	Sample Identification	Iso Rerun?	Iso Breaks?	Dalt Breaks?
a	1				|—·—·—·—|	❑
b	2				|—·—·—·—|	❑
c	3				|—·—·—·—|	❑
d	4				|—·—·—·—|	❑
e	5				|—·—·—·—|	❑
f	6				|—·—·—·—|	❑
g	7				|—·—·—·—|	❑
h	8				|—·—·—·—|	❑
i	9				|—·—·—·—|	❑
j	10				|—·—·—·—|	❑
k	11				|—·—·—·—|	❑
l	12				|—·—·—·—|	❑
m	13				|—·—·—·—|	❑
n	14				|—·—·—·—|	❑
o	15				|—·—·—·—|	❑
p	16				|—·—·—·—|	❑
q	17				|—·—·—·—|	❑
r	18				|—·—·—·—|	❑
s	19				|—·—·—·—|	❑
t	20				|—·—·—·—|	❑

Comments:

17 Preparing Dalt Slab Gel Numbers

Slab gel numbers are prepared by typing onto Whatman #1 filter paper. This is most easily done by setting up a matrix of gels numbers in a word processing program, and then printing this onto filter paper either with an impact printer that uses a carbon ribbon, or with a laser printer. The filter paper must be cut rectangular to go through the printers. Although we do it routinely, we are not responsible for whatever happens to your laser printer when it's fed filter paper...

Use one set (Xnna-Xnnz) for each batch of slab gels (see subsections 6.1.3 and 6.3.1). Cut out the set as a strip, then trim the excess and begin cutting the numbers apart. Keep them in order by cutting them off as you move along the bench top and try to have them land face up. Then pick up the individual numbers one at a time and drop them into successive Dalt cassettes (in the casting box). You should only need a-v (22 cassettes).

Once the gel is polymerized in the cassettes, each number will be an integral part of its gel. They will be at the bottom where they do not disturb the proteins' migration and will be visible after staining. They thus serve to identify the gel through all subsequent steps.

18 ISO-DALT® User's Group and Sources of Equipment

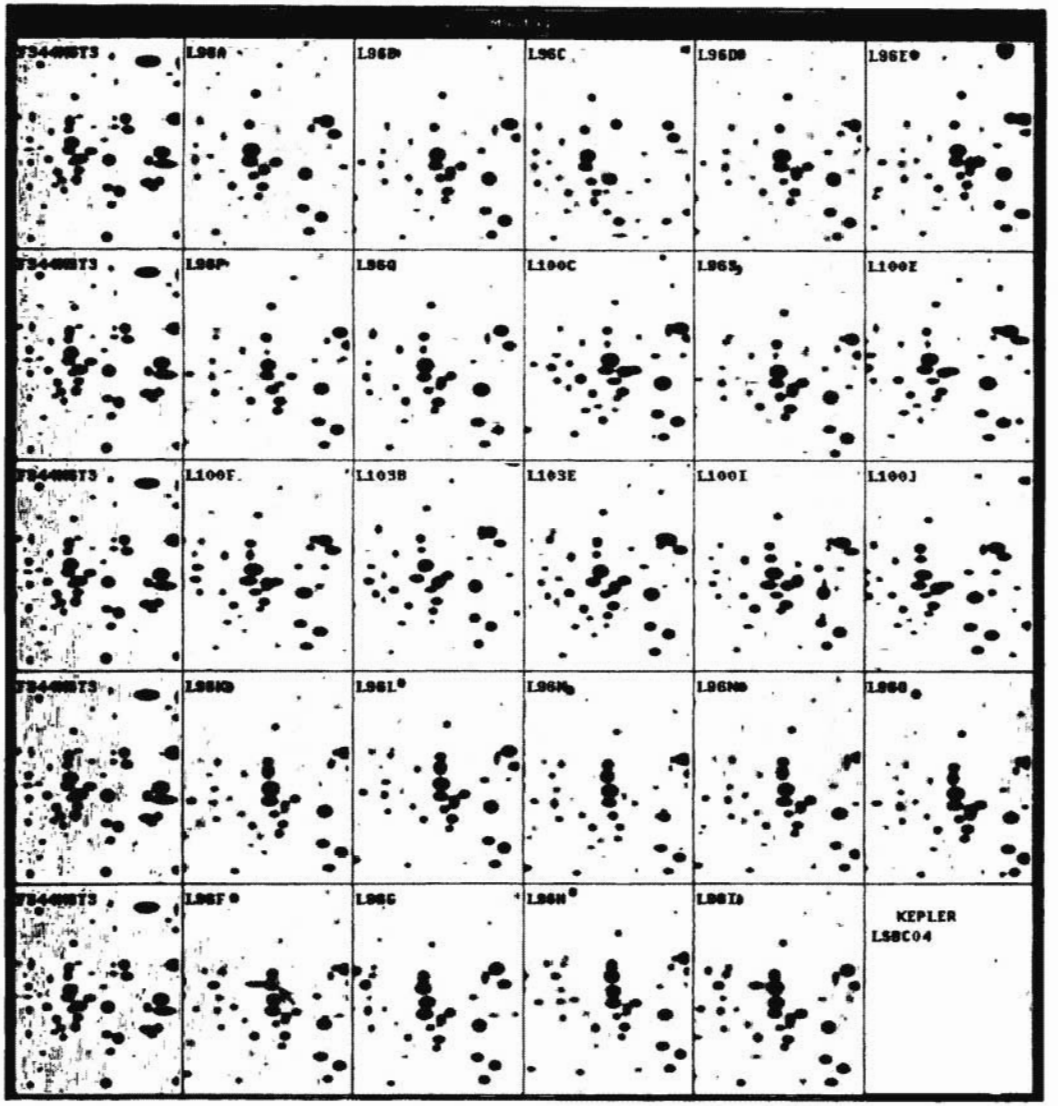
LSB periodically distributes an ISO-DALT User's Group Newsletter for those with an interest in trading technical tips and generally keeping up with 2-D technology. If you would like to be included in the User's Group and receive the Newsletter, write to the address below. There is no charge.

ISO-DALT User's Group
Large Scale Biology Corporation
9620 Medical Center Drive
Rockville, MD 20850
Telephone: (301) 424-5989
Fax: (301) 762-4892

The ISO-DALT equipment referred to in this manual can be obtained exclusively from Hoefer Scientific and their overseas distributors.

Hoefer Scientific Instruments
654 Minnesota Street
San Francisco, CA 94107
Toll free: (800) 227-4750
In CA: (415) 282-2307
Fax: (415) 821-1081
Telex: 470778

For information on Angelique, Wedgies, the Equalizer, or the Kepler computer workstation for analysis of 1- and 2-D gels, or on LSB's databases of 2-D gel-related information, contact Large Scale Biology in Rockville.



Cover Art:

Front: Coomassie blue-stained ISO-DALT® 2-D pattern of rat liver homogenate.

Above: Montage of regions from 2-D gels showing regulation of rat liver HMG-CoA reductase (arrow) *in vivo* by various drug treatments. A single master pattern (left column) is replicated for each experimental group (row).

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