

Gel Electrophoresis of Nucleic Acids:

a practical approach

Edited by:

D. Rickwood and B. D. Hamer

Gel Electrophoresis of Nucleic Acids:

a practical approach

Edited by:

D. Rickwood

Department of Biology, University of Essex,
Colchester, Essex, England

B. D. Hames

Department of Biochemistry,
University of Leeds, Leeds, England

ISBN 0-904-14724-X



Published by IRL Press Limited, Oxford and Washington DC

IRL Press Ltd,
P.O.Box 1,
Eynsham,
Oxford OX8 1JJ
England.

© 1982 IRL Press Limited

All rights reserved by the publisher. No part of this book may be reproduced or transmitted in any form by any means, electronic or mechanical, including photocopying, recording or any information storage and retrieval system, without permission in writing from the publisher.

British Library Cataloguing in Publication Data

Gel electrophoresis of nucleic acids.

1. Nucleic acids 2. Electrophoresis – Laboratory manuals

I. Rickwood, D. II. Hames, B.

547'.596 QD433

ISBN 0-904-14724-X

Cover Photograph

A typical DNA sequencing gel autoradiograph as described in chapter 4.

Printed in England by Information Printing Limited, Eynsham.

Preface

The development of separation methods has played a significant role in the elucidation of biological systems. Of the various techniques in common use, one of the most important is gel electrophoresis. This book and its companion volume (see back cover for contents) are designed to provide details of gel electrophoretic procedures for the separation of macromolecules. The main emphasis of each book is on the practical aspects of the electrophoretic techniques in current use. Several revisions of some chapters were necessary in order to prevent undue repetition whilst including important practical topics and we thank the authors concerned and particularly the publishers for their patience and understanding during this exercise.

D.Rickwood and B.D.Hames

Contributors

Albert E. Dahlberg

Brown University, Providence, Rhode Island 02912, USA.

James M. D'Alessio

Bethesda Research Laboratories Inc., P.O. Box 577, Gaithersburg, Maryland 20760, USA.

R. Wayne Davies

Department of Biochemistry, UMIST, P.O. Box 88, Manchester M60 1QD, U.K.

Rupert De Wachter

Department of Cell Biology, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium.

Walter Fiers

Laboratory of Molecular Biology, Rijksuniversiteit-Gent, Ledeganckstraat 35, B-9000 Gent, Belgium.

Graham H. Goodwin

Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, London SW3 6JB, U.K.

Don Grierson

Department of Physiology and Environmental Studies, School of Agriculture, University of Nottingham, Sutton Bonington, Loughborough LE12 5RD, U.K.

Stephen J. Minter

Department of Biochemistry, UMIST, P.O. Box 88, Manchester M60 1QD, U.K.

Paul G. Sealey

MRC Mammalian Genome Unit, Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, U.K.

Ed. M. Southern

MRC Mammalian Genome Unit, Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, U.K.

IMPORTANT NOTICE

HEALTH HAZARDS OF GEL ELECTROPHORESIS

- (i) A number of chemicals commonly used for gel electrophoresis are toxic whilst the status of others remains unknown. It is very important that experimenters acquaint themselves with the precautions required for handling all chemicals mentioned in this text. Particular care should be taken when handling acrylamide since this is a known potent neurotoxin. Polyacrylamide gel is not toxic unless it contains unpolymerised monomer.
- (ii) Care should be taken when using gel electrophoresis apparatus that no electrical safety hazard exists. Particular care should be taken when using apparatus not obtained from commercial sources since this may not meet the usual required safety standards. It is recommended that all apparatus is checked by a competent electrician before use.

Abbreviations

A ₅₄₀	absorption at 540 nm
A	adenine (only used as part of a sequence)
A	amp(s), ampere(s)
ABM-paper	aminobenzyloxymethyl-paper
AC	alternating current
% acrylamide	polyacrylamide gel concentration expressed in terms of <i>total</i> monomer (i.e. acrylamide and crosslinker)
AraCTP	arabinosyl CTP
BAC	<i>N,N'</i> bisacrylylcystamine
Bisacrylamide	<i>N,N'</i> -methylene bisacrylamide
bp	base pairs
Bq	becquerel (1 disintegration/sec)
C	cytosine
cDNA	complementary DNA
Ci	Curie (3.7×10^{10} Bq)
cpm	counts per minute (the exact amount of radioactivity depends on the isotope and the method of measurement used)
% crosslinker	expressed in terms of <i>total</i> monomer (i.e. acrylamide and crosslinker)
CTAB	cetyltrimethylammonium bromide
DATD	<i>N,N'</i> diallyltartardiamide
DBM-paper	diazobenzyloxymethyl-paper
DC	direct current
ddH ₂ O	double distilled water
ddNTP	dideoxynucleotide triphosphate
DMAPN	3-dimethylamino-propionitrile
DMS	dimethyl sulphate
DMSO	dimethyl sulphoxide
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dpm	disintegrations per minute (60 Bq)
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetate
G	guanine
g	gram(me)
× g	centrifugal force (× unit gravitational field)
h	hour(s)
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulphonic acid
HTAB	hexadecyl-trimethyl ammonium bromide
i.d.	internal diameter
IPTG	isopropylthio-β-D-galactoside
kb	kilobases
kBq	kilobecquerels (Bq × 10 ³) see Bq
kV	kilovolts

M	molarity
<i>M</i>	mobility
mA	milliamp(s)
MBq	megabecquerels ($\text{Bq} \times 10^6$) see Bq

Contents

HEALTH WARNING

ABBREVIATIONS

1. GEL ELECTROPHORESIS OF RNA	1
Don Grierson	
Introduction	1
Polyacrylamide Tube Gel Electrophoresis	2
Apparatus	2
Preparation of gels	2
Setting up the electrophoresis apparatus	3
Sample preparation and loading of the gels	4
Electrophoresis of gels	6
Scanning the gels	8
Slicing of gels	9
Measurement of radioactivity in gel slices	10
Elution and recovery of RNA	10
Modifications to the Basic Tube Gel Method	12
Removal of DNA prior to electrophoresis	12
Denaturation of RNA before loading	13
Running gels at non-ambient temperatures	13
Gradient gels	14
Solubilisable polyacrylamide gels	14
Agarose-acrylamide composite gels	14
Other electrophoresis buffers	14
Denaturing Gels	15
Formamide gels	16
Urea gels	17
Methyl mercuric hydroxide gels	17
Molecular Weight Estimations	17
Non-denaturing gels	17
Denaturing gels	19
Electrophoresis in Polyacrylamide Slab Gels	19
Apparatus	19
Recipes for gels	21
Casting the gels	24
Electrophoresis	24
Detection and recovery of RNA in slab gels	24
Transfer of RNA to diazobenzyloxymethyl-paper for hybridisation analysis	29
Problems of Polyacrylamide Gel Electrophoresis and their Remedies	31
Purification of Histone Messenger RNA: A Case Study	36
References	38

2. ELECTROPHORESIS OF DNA	39
Paul G. Sealey and Ed M. Southern	
Introduction	39
Basic Techniques and Their Application	39
Equipment	40
Buffers for non-denaturing gels	41
Buffers for denaturing gels	43
Gel preparation	43
Sample preparation and loading of the gels	46
Size markers for gels	48
Electrophoresis conditions	49
Analysis of gels after electrophoresis	49
Recovery of DNA fragments from agarose gels	53
Analysis of DNA fragments using hybridisation	55
Large Scale Preparative Gel Electrophoresis of DNA	59
The annular preparative apparatus	59
The linear preparative apparatus	67
Examples of fractionations	73
Acknowledgements	75
References	75
 3. TWO-DIMENSIONAL GEL ELECTROPHORESIS OF NUCLEIC ACIDS	 77
Rupert De Wachter and Walter Fiers	
Introduction to Two-dimensional Gel Electrophoresis of RNA	77
Factors governing the electrophoretic mobility of RNA on polyacrylamide gels	77
Main types of two-dimensional gel systems	79
Apparatus and Experimental Procedure for Two-dimensional Separations of RNA	81
Separation in the first dimension	81
Separation in the second dimension	86
Variations and modifications of the procedure	88
Temperature control	89
Auto-radiography	89
Detection of non-radioactive RNA	90
Measurement of radioactivity in excised slices	90
Recovery of RNA from the gel	91
Examples of Two-dimensional Separations of RNA	92
Separation of oligonucleotides	92
Separation of RNA fragments	95
Separation of small RNA molecules	98
Separation of mRNAs	101

Introduction to Two-dimensional Gel Electrophoresis of DNA	105
Separation of DNA Restriction Fragments on Conventional Gels	106
Basis of the separation	106
Separation in the first dimension	106
Separation in the second dimension	108
Examples of the application of the technique: separation of viral DNA restriction fragments	109
Separation of DNA Restriction Fragments on Denaturing Gradient Gels	110
Basis of the separation	110
Apparatus	111
Separation in the first dimension	112
Separation in the second dimension	112
Staining and autoradiography of gels	115
Examples of application of the technique: separation of bacterial DNA fragments	115
Acknowledgements	115
References	115
4. DNA SEQUENCING	117
R. Wayne Davies	
Introduction	117
DNA Sequencing by the Chain Terminator Method	120
Equipment and materials	120
Manipulation of small volumes of liquid	120
Characteristics of primer fragments	123
Isolation of DNA fragments	123
Preparation of single-stranded template DNA	128
Annealing of primer and template	130
The primed DNA synthesis reaction	132
Ribosubstitution: an alternative way of removing primer fragments	136
Application of the chain terminator method to the determination of DNA sequences <i>within</i> DNA fragments	137
Making Polyacrylamide Gels for DNA Sequencing	139
Equipment	139
Materials	141
Preparing the gel mould	141
Stock solutions	142
Pouring the gel	143
Preparation of the gel for electrophoresis	144
Loading the samples	144
Electrophoresis	145
Preparing the gel for autoradiography	145
Autoradiography	146
Reading and Interpreting Autoradiographs of Chain Terminator Sequencing Experiments	146

Common problems in interpreting gel autoradiographs	148
The M13 Sequencing System	149
Characteristics of M13 bacteriophage	149
The M13 cloning system	150
Using the M13 cloning system	151
Chain terminator sequencing with the M13 system	155
DNA Sequencing by the Chemical Method	159
The basic steps of the procedure	159
Equipment and materials	159
Input and purification of DNA fragments	161
End-labelling DNA fragments	161
Isolation of DNA fragments labelled at one end only	166
The base-specific cleavage reactions	166
Electrophoresis of the reaction products	171
Reading and interpreting autoradiographs of Maxam-Gilbert sequencing gels	171
Use of Computers	171
References	172
5. RNA SEQUENCING	173
James M. D'Alessio	
Introduction	173
The Isolation of RNA for Sequencing	174
Handling Microgram Quantities of RNA	175
Precautions against ribonuclease	175
Reaction vessels	176
Ethanol precipitation of RNA	176
General Comments on Terminally-labelled RNA Molecules for Sequencing	176
Labelling 5' termini	176
Labelling 3' termini	177
Preparative gel electrophoresis	177
Preparation of Terminally-labelled RNA for Sequencing	178
Removal of the cap structure	178
Labelling the 5' terminus with [γ - ^{32}P]ATP and T4 polynucleotide kinase	179
Labelling the 3' terminus with [5'- ^{32}P]pCp and T4 RNA ligase	180
Purification of ^{32}P -RNA by polyacrylamide gel electrophoresis	181
Elution of ^{32}P -RNA from polyacrylamide gels	182
Specific Cleavage of RNA for Sequencing	183
Fragmentation strategy using ribonuclease H	183
Site specific cleavage of RNA	184
Base Specific Cleavage of RNA: Enzymatic Sequencing	185
General comments	185
Procedures for the enzymatic sequencing of RNA	185
Alkaline hydrolysis of RNA	186

Base Specific Cleavage of RNA: Chemical Sequencing	187
General comments	187
Procedures for the chemical sequencing of RNA	188
RNA Sequencing Gels	191
Important considerations	191
Casting a thin sequencing gel	192
Autoradiography of sequencing gels	193
Interpretation of the autoradiographs	194
References	196
6. ELECTROPHORESIS OF NUCLEOPROTEINS	199
Graham H. Goodwin and Albert E. Dahlberg	
Electrophoresis of Nucleosomes	199
Introduction	199
Equipment	199
Stock solutions	201
Preparation of the sample	202
Preparation of the polyacrylamide gel slab	202
Sample loading and electrophoresis	204
Detection of the nucleosomal bands	204
Extraction of nucleosomal particles from the gels	206
Recovery and analysis of the protein and DNA of the extracted nucleosomes	207
Possible problems and their remedies	209
Two-dimensional electrophoresis of nucleosomes	210
Electrophoresis of Ribosomes and Polysomes	213
Introduction	213
Equipment	214
Stock solutions	214
Preparation of the sample	215
Preparation of agarose-acrylamide composite gels	215
Sample loading and electrophoresis	217
Gel-staining	217
Two-dimensional gel electrophoresis	218
Applications of different composite gels	218
Possible problems and their remedies	224
References	224
APPENDIX I. Nucleic Acid Molecular Weight Markers	227
APPENDIX II. Suppliers of Specialist Items for Electrophoresis	233
INDEX	237

CHAPTER 1

Gel Electrophoresis of RNA

DON GRIERSON

1. INTRODUCTION

The term electrophoresis is generally applied to the movement of small ions and charged macromolecules in solution under the influence of an electric field. The rate of migration depends on the size and shape of the molecule, the charge carried, the applied current and the resistance of the medium. Zone electrophoresis is the separation of charged molecules in a supporting medium, resulting in the migration of charged species in distinct zones and is distinguished from boundary electrophoresis which is carried out in free solution.

Successful fractionation of RNA by electrophoresis was achieved in the middle of the 1960s and very good separations became routine by the end of that decade. The chief reasons for success were improvements in RNA preparation and handling techniques and the introduction of supporting gels for electrophoresis. The basic gel technology arose by trial and error without complex theory. In an electric field, at moderate pH, negatively-charged RNA migrates towards the anode. A fractionation is achieved because large molecules move more slowly through the gel than small molecules and separation of RNA within a given size range is obtained by selecting a gel of appropriate pore size.

Electrolytes used in electrophoresis generally consist of an aqueous buffer, containing a chelating agent such as ethylenediaminetetraacetate (EDTA) and a nuclease inhibitor such as sodium dodecyl sulphate (SDS). For some purposes it is important to reduce the effects of secondary structure and a denaturing agent may be added or used in place of water as solvent. A number of factors affect the fractionation of RNA. Increasing the current leads to higher rates of migration, but the flow of current also results in the production of heat, which, if excessive, adversely affects the separation by causing trailing and broadening of the zones. Increasing the ionic strength results in lower mobilities. If the current is increased to compensate, this again may result in adverse effects due to heating. On the other hand, if the ionic strength is too low, this may seriously reduce the buffering capacity of the solution and lead to undesirable pH changes.

Electrophoresis is carried out in gels cast either in tubes or as slabs. A number of gel materials have been used successfully, including agar, agarose and polyacrylamide. Agar and agarose gels are made by heating the granular material in the appropriate electrolyte buffer, casting the gels and allowing them to set on cooling. The resolving power of these gels depends on the concentration of dissolved agar or agarose; dilute gels are used for very large RNA molecules and more concentrated gels for low molecular weight RNA.

Polyacrylamide gels are made from acrylamide and *N,N'*-methylene bisacrylamide (bisacrylamide) mixtures dissolved in electrolyte and polymerised by the addition of chemical catalysts. The physical properties and resolving power of the gels are determined by the acrylamide concentration and the proportion of bisacrylamide added as crosslinker. Examples of the resolution obtainable with different concentrations of polyacrylamide in tubes and slab gels are given later in this chapter (Figures 2, 11, 12, and 16). The wide range of controlled pore size which can be reproducibly obtained with polyacrylamide makes this a very popular choice and gels can be made for most purposes. However, some researchers find very dilute polyacrylamide gels, used for fractionating very large RNAs, difficult to handle and either strengthen them with agarose or use agarose on its own.

In many cases, the distance moved by an RNA molecule during electrophoresis is inversely related to the \log_{10} molecular weight. However, base composition, secondary structure and other factors may change this relationship. For accurate measurement of molecular weights it is necessary to carry out electrophoresis under completely denaturing conditions, for example by the inclusion of methyl mercuric hydroxide in the gel system or by electrophoresis in the presence of formamide. Both of these techniques are discussed later.

It is not possible to review all the individual variations that are used in the gel electrophoresis of RNA. Therefore this chapter describes some of the most frequently used methods and their limitations. The method finally chosen is often dictated by one's own requirements and may be improved by experience.

2. POLYACRYLAMIDE TUBE GEL ELECTROPHORESIS

2.1 Apparatus

Polyacrylamide gel electrophoresis in tubes is convenient for the analysis of a relatively small number of samples or where small quantities of RNA are to be fractionated, although it can be scaled up as a preparative procedure. Table 1 lists the equipment that is required. Suitable electrophoresis apparatus and gel tubes (Figure 1) can be easily and inexpensively constructed in a workshop or purchased as complete units from any suppliers. Perspex sheets 3 mm thick are suitable for making buffer reservoirs; the Perspex sheets can either be glued or fused together with chloroform. The electrodes should be made of platinum wire. Perspex tubing cut to the required length is the best material for gel tubes; glass tubes are not really suitable because the gels stick to the glass and so are difficult to recover for analysis.

2.2 Preparation of Gels

Recipes for making gels from 2–10% polyacrylamide are given in Tables 2 and 3 and illustrations of the resolving power of 2.4%, 3% and 10% gels are shown in Figure 2. Acrylamide is toxic and should not be inhaled, swallowed or allowed to contact the skin. The effects are cumulative.

Before preparation of the gels, the tubes should be temporarily sealed at the base and held vertically in a rack or in the electrophoresis apparatus. Parafilm, rubber bungs or caps can be used to seal the base of the gel tubes. Another method is to cut a transverse section of flexible plastic tubing to fit the inside of the tube and then to

Table 1. Equipment Required for Polyacrylamide Gel Electrophoresis in Tubes.*Electrophoresis*

1. Electrophoresis apparatus made from Perspex (see *Figure 1*).
2. Gel tubes, preferably made from Perspex. These can be of any convenient dimensions, such as 0.6 cm or 0.9 cm internal diameter tubing cut to 8–12 cm lengths. With larger diameter tubes, heat dissipation during electrophoresis is less efficient and this may cause problems.
3. A good vacuum pump.
4. A power pack providing up to 500 V and 100 mA and the facility for constant voltage. It is not necessary to provide constant current.

Scanning of gels

5. Gel scanning equipment. Several types are available, such as a "Polyfrac" made by Joyce-Loebl Ltd. or a spectrophotometer fitted with a gel-scanning attachment. These normally require a parallel-sided quartz glass cuvette or a tube to which the gels are transferred after electrophoresis. Alternatively, the gels may be run in quartz glass tubes and scanned *in situ* but these tubes are expensive.

Measurement of radioactivity

6. Gel slicer. Accurate slicing of dilute gels (2.2% or 2.4%) is difficult unless they are first frozen and then sliced using an automated gel slicer (e.g., from Joyce-Loebl Ltd.). More concentrated gels can be sliced unfrozen, but with less precision, using razor blades.
7. Scintillation counter or planchette counter.

plug the hole with a short piece of glass rod. A stock solution of acrylamide and bisacrylamide is diluted to the appropriate concentration with gel buffer and water (*Table 3*), and dissolved oxygen, which inhibits polymerisation, is removed using a vacuum pump for approximately 30 sec. The vacuum should be good enough to cause the solution to bubble vigorously. Gels will polymerise even if oxygen is not removed, but with low concentrations of acrylamide this often results in weak or uneven gels. It is also difficult to polymerise gels with similar properties from day to day without degassing. The tetramethylethylenediamine (TEMED) and ammonium persulphate (the polymerisation catalysts) are added after degassing, the solution is gently mixed and immediately pipetted into the tubes. The gel mixture should then be carefully overlaid with water to a depth of about 1 cm using a Pasteur pipette with a curved tip or a syringe fitted with a bent needle. This produces a flat surface at the top of the gel and also prevents the gel from drying out before use. Most gels are ready to use within 2 h although they continue to polymerise slowly if left longer. They can be stored for a few days at room temperature or in a refrigerator. Gels below approximately 2.6% polyacrylamide are almost transparent whereas more concentrated gels are progressively more translucent to visible light.

2.3 Setting up the Electrophoresis Apparatus

It takes about half an hour to get the gels ready for loading the samples. The gel tubes should be arranged vertically by pushing them through the rubber grommets in the upper electrophoresis reservoir. The water overlay is removed from the tops of the gels using a Pasteur pipette or by a flick of the wrist and the stoppers are then removed from the bottom of the gel tubes. Very dilute gels may slide out of the tubes unless they are supported by a small piece of muslin held in place over the

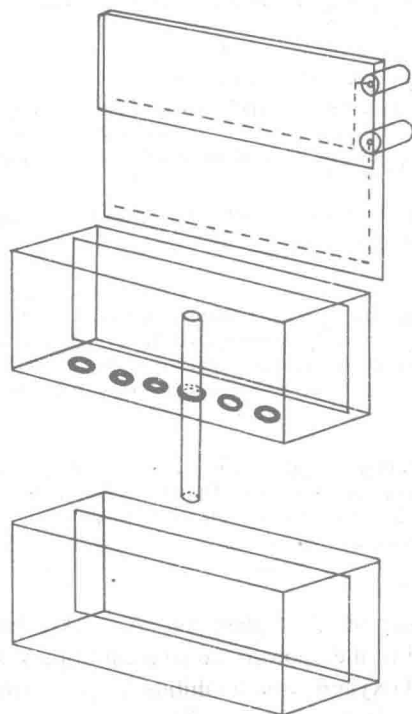


Figure 1. A tube gel electrophoresis apparatus. This design, which is used in many laboratories, can be constructed from 3 mm thick Perspex sheet. It consists of two rectangular buffer reservoirs plus electrodes. The upper reservoir rests on the lower reservoir; gel tubes pass through holes drilled in the base of the upper reservoir and are held in position by rubber grommets. The one-piece electrode assembly, made from Perspex sheet and platinum wire, has two limbs which fit into the upper and lower reservoirs. The dimensions of the electrophoresis apparatus depend on the size and number of gel tubes. The gel tubes are shielded from the electrodes to localise pH changes and the products of electrolysis.

bottom of the tube with a rubber band. A plastic ring in the bottom of the gel tube will also prevent all but the most dilute gels from slipping out. The upper and lower electrophoresis reservoirs are then filled with sufficient reservoir buffer (*Table 2*) to cover the tops of the gels, and the upper reservoir is placed on top of the lower compartment. It is important to remove any air bubbles trapped at the bottom of each gel tube. This is easily done by directing a jet of buffer at the base of the gel with the curved tip of a Pasteur pipette. This technique can also be used to remove air bubbles from the top of gels. The electrodes are then placed in position (lower electrode positive) and the power pack is switched on. Electrophoresis may be carried out at room temperature or in a cold room (5°C). It is normal practice to pre-run the gels at approximately 5 V/cm for 15–30 min to remove catalysts and UV-absorbing materials before loading the sample.

2.4 Sample Preparation and Loading of the Gels

RNA frequently tends to aggregate at the top of the gel if it is contaminated with proteins but normally this presents no problems since most procedures for RNA