Gel Electrophoresis of Nucleic Acids:

a practical approach

Edited by:
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Cover Photograph
A typical DNA sequencing gel autoradiograph as described in chapter 4.

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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.

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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 *total* monomer (i.e. acrylamide and crosslinker)
AraCTP	arabinosyl CTP
BAC	*N,N'* bisacrylylcystamine
Bisacrylamide	*N,N'*-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 *total* monomer (i.e. acrylamide and crosslinker)
CTAB	cetyltrimethylammonium bromide
DATD	*N,N'* diallyltartardiamide
DBM-paper	diazobenzyloxymethyl-paper
DC	direct current
ddH_2O	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)
$\times$ g	centrifugal force ($\times$ unit gravitational field)
h	hour(s)
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HTAB	hexadecyl-trimethyl ammonium bromide
i.d.	internal diameter
IPTG	isopropylthio-β-D-galactoside
kb	kilobases
kBq	kilobecquerels ($Bq \times 10^3$) see Bq
kV	kilovolts

M	molarity
M	mobility
mA	milliamp(s)
MBq	megabecquerels (Bq $\times 10^6$) see Bq

Contents

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 overlayered 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

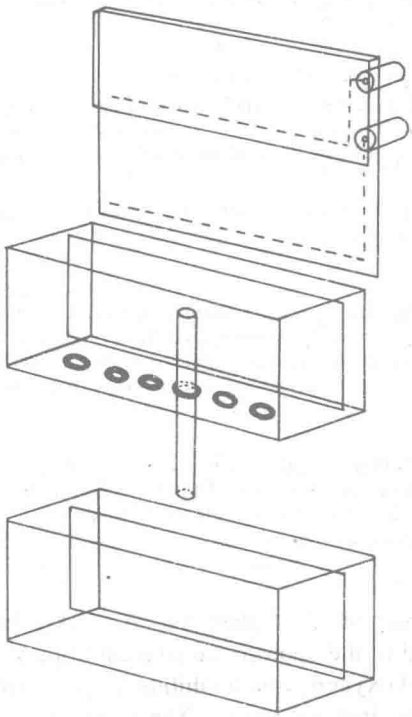

Figure 1. A tube gel electrophoresis apparatus. This design, which is used in many laboratories, can be constructed from 3 mm thick Perspex sheet. It consists of two rectangular buffer reservoirs plus electrodes. The upper reservoir rests on the lower reservoir; gel tubes pass through holes drilled in 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