height of the flash unit or by adding or deleting filters. The flashed face of the film should face the fluor, in the sample for $^{35}S$, in the screen for $^{32}P$.

### Quantitation of Autoradiographs

Film response to autoradiography (no fluors or screens) is linear up to absorbances of about 1 unit. With $^{35}S$ autoradiography, it is important that the gel matrix be uniform, because different regions of a gradient gel could quench $^{35}S$ to different extents. Even though the nonlinear film response in fluorography can be corrected by preflashing the film, linearity should be checked with standards prepared with the same isotope.

## [8] Electrophoresis in Agarose and Acrylamide Gels

*By* RICHARD C. OGDEN and DEBORAH A. ADAMS

The scope of this chapter is to present a range of methods by which DNA and RNA molecules can be fractionated and analyzed by means of gel electrophoresis. This chapter will emphasize those techniques which can be simply and routinely applied in the course of molecular cloning and analysis and, wherever appropriate, reference will be made to more exhaustive practical or theoretical considerations of the techniques.

Gel electrophoresis through agarose or polyacrylamide is a very powerful method for rapidly resolving mixtures of nucleic acid molecules which has found wide application in recombinant DNA research. The resolution afforded far exceeds that generally obtained by other sizing techniques. The fractionated nucleic acids can be directly "viewed" *in situ* in the gel and can be readily recovered by a variety of methods tailored to subsequent steps in an experimental protocol. Because it is such an indispensible technique, a great deal of effort has gone into improving its efficacy for particular applications, with the result that many of the original methods have been simplified, scaled down, and improved. This review will cover the various choices to be made when confronted with experiments requiring gel electrophoresis in the order they would typically arise, starting with the type of gel system, choice of running buffer, and equipment, and continuing through gel running, visualization of the separated molecules, extraction of the material from the gel, and workup of preparative samples. Certain specific applications of electrophoresis, for example gels for sequencing purposes (this volume [56, 57])

and transfer of nucleic acids from gels to membranes (this volume [45, 61]), will be covered in separate chapters dealing with these specific techniques.

## Gel Electrophoresis of DNA

Gel electrophoresis, as applied to the fractionation of DNA, is a very versatile technique and methods exist for the fractionation of single- or double-stranded molecules ranging in size from a few bases to chromosome-sized duplexes. With an emphasis on the practical application of the technique to molecular cloning, this section will cover the most common usages. DNA may be fractionated in agarose or polyacrylamide gels. Low percentage acrylamide gels are occasionally strengthened by agarose to yield a composite gel which resolves solely according to the acrylamide percentage.

At pH near neutrality, DNA is negatively charged and migrates from cathode to anode with a mobility dependent primarily on fragment size. Normally, smaller linear DNA fragments migrate faster than larger ones. Nondenaturing polyacrylamide gels can be used for separation of double-stranded DNA fragments between 6 bp (20% acrylamide) and 1000 bp (3% acrylamide). Nondenaturing agarose gels can be used for fragments between 70 bp (3% agarose) and 800,000 bp (0.1% agarose). Pulse-field electrophoresis for separation of larger DNA duplexes will be considered later. As little as 1 ng per band of nonradioactive double-stranded sample can be detected (in agarose gels) and both systems are readily amenable to cloning-scale (microgram amounts) preparative work.

Single-stranded DNA can be fractionated by agarose or polyacrylamide gel electrophoresis by inclusion of a denaturing reagent in the gel. There are several denaturing systems available but because of incompatibility between the gel material and denaturant, certain combinations are precluded. The choice of denaturing gel system depends not only on the size of fragments to be resolved but also on the experimental protocol involving the desired fragment after purification. Fractionation of single-stranded DNA can also be achieved by a reversible pretreatment of the sample with glyoxal prior to running the gel at neutral pH. The options available for particular cases will be discussed in the relevant sections below. A more detailed review of gel electrophoresis of DNA with an emphasis on practical approaches has been published.[1]

[1] P. G. Sealey and E. M. Southern, *in* "Gel Electrophoresis of Nucleic Acids—A Practical Approach" (D. Rickwood and B. D. Hames, eds.), p. 39. IRL Press, Washington, D.C., 1982.

## Gel Electrophoresis of RNA

Most of the strengths of gel electrophoresis as a technique for fractionating DNA apply equally to RNA. As much of the RNA encountered in the course of molecular cloning is single-stranded, any of the applications of the technique to RNA involve the use of denaturing gels or two-dimensional gels in which the pH or denaturant concentration is varied between the two dimensions such that the structure of the molecules changes. A detailed review of two-dimensional gel electrophoresis techniques has been published.[2]

Agarose and polyacrylamide slab gels are most frequently used for RNA work and the types of gel selected are very similar to those for DNA of comparable size and complexity. It is important to stress that gel electrophoresis of single-stranded RNA, in particular mRNA, should be carried out with equipment and reagents that have been purged of nucleases. For this reason, many laboratories consider it prudent to keep certain electrophoresis equipment for RNA use only.

## Choice of Gel System

For the most part, the size range of the sample will serve to determine the choice of gel for both analytical and preparative work. As a generalization, agarose is used for larger molecules, and polyacrylamide for shorter. Low percentage polyacrylamide gels, strengthened with agarose,[3] are used to extend the useful range of polyacrylamide gels.

There is an intermediate size range (70–1000 bp for double-stranded DNA) for which the choice of polyacrylamide or agarose is available. For analytical work, the prime considerations will be the rapidity and ease of the technique, and for this reason the horizontal agarose minigel is widely preferred. For preparative work, polyacrylamide offers many advantages, principally ease of elution and lack of coeluted inhibitors of subsequent enzymatic reactions, but the introduction of inhibitor-free low-melting temperature agarose (see later) has circumvented many earlier problems associated with preparative agarose gel electrophoresis.

The choice of denaturing gel system is determined principally by the aims of the experiment. Denaturants such as formamide, formaldehyde, and methylmercuric hydroxide are toxic and common sense dictates that

[2] R. DeWachter and W. Fiers, *in* "Gel Electrophoresis of Nucleic Acids—A Practical Approach" (D. Rickwood and B. D. Hames, eds.), p. 77. IRL Press, Washington, D.C., 1982.

[3] A. C. Peacock and C. W. Dingman, *Biochemistry* **7**, 668 (1968).

their use is restricted to essential applications. For many purposes, other less hazardous denaturants such as alkali or urea, or pretreatment with glyoxal can be used without compromising the purpose of the experiment.

## Polyacrylamide Gel Electrophoresis of DNA and RNA

Polyacrylamide gels result from the polymerization of acrylamide monomers into linear chains and the linking of these chains with *N,N'*-methylenebisacrylamide (bis). The concentration of acrylamide and the ratio of acrylamide to bis determine the pore size of the resultant three-dimensional network and hence its sieving effect on nucleic acids of different sizes. The effective range of separation for different concentrations of acrylamide is shown in Table I together with the approximate size of duplex DNA which would comigrate with the marker dyes bromphenol blue and xylene cyanole FF.[4] Two denaturants are commonly used in conjunction with polyacrylamide for the fractionation of single-stranded nucleic acids, urea (final concentration 7 *M*) or formamide (98%). The preparation and running of denaturing polyacrylamide gels have been discussed previously in this series[5] but a brief outline will be presented here for the sake of completeness. The choice of denaturant is dictated largely by the size of the molecule. Urea is commonly used for sizing and resolving smaller chains (up to ~200 nucleotides). Larger chains and those containing substantial self-complementarity will retain secondary structure in urea, and 98% formamide will be necessary for accurate sizing and good resolution. Thin, urea-containing gels (discussed in the chapters on DNA and RNA sequencing [56,57]) can be run at elevated temperatures, thereby increasing their useful range.

Typical applications in which polyacrylamide gels would be the method of choice include purification of synthetic oligonucleotides, preparative isolation or analysis of DNA (<1 kb), especially if labeled, and resolution of small RNA molecules (e.g., tRNA) by two-dimensional techniques.

### *Equipment*

Polyacrylamide gels of all kinds are invariably poured between two glass plates separated by spacers and run in the vertical position. A typical preparative gel will use 1- to 2-mm spacers and a 0.5- to 1-cm comb. The capacity of such a well is approximately 1 $\mu$g per band. As a general rule however, thinner gels (0.5 mm) are preferred since efficient elution by

[4] T. Maniatis, A. Jeffrey, and H. V. Van de Sande, *Biochemistry* **14**, 3787 (1975).
[5] T. Maniatis and A. Efstratiadis, this series, Vol. 65, p. 299.

TABLE I
RANGE OF SEPARATION OF ACRYLAMIDE GELS

| Acrylamide (% w/v) (acrylamide : bis, 29 : 1) | Optimal range of separation (bp) | Comigration sizes (bp) | |
|---|---|---|---|
| | | Bromphenol blue | Xylene cyanole |
| 3.5 | 100–1000 | 100 | 460 |
| 5.0 | 80–500 | 65 | 260 |
| 8.0 | 60–400 | 45 | 160 |
| 12.0 | 40–200 | 20 | 70 |
| 20 | 6–100 | 12 | 45 |

diffusion is possible without crushing, leading to less contamination with acrylamide. Any commercially available apparatus for vertical slab gels can be used and examples of cheaper, homemade apparati abound.[6-8] Many research establishments have access to a machine shop capable of working with Plexiglas to make spacers, combs, and gel stands.

*Running Buffers*

The best and most common choice of running buffer for nondenaturing or urea-containing denaturing polyacrylamide gels is Tris–borate–EDTA (TBE), pH 8.3.[3] This is commonly stored as a 10× stock solution. Denaturing gels containing formamide are most conveniently run in phosphate buffer.

*Nondenaturing Running Buffer*

Concentrated stock solution (10× TBE):

| | |
|---|---|
| Tris base | 108 g |
| Boric acid | 55 g |
| Disodium EDTA · $2H_2O$ | 9.3 g |
| Water to | 1 liter |
| (The pH should be 8.3) | |

Working solution (1×):

0.089 *M* Tris–borate, pH 8.3
0.025 *M* Disodium EDTA

[6] T. Maniatis, E. E. Fritsch, and J. Sambrook, "Molecular Cloning—A Laboratory Manual," p. 175. Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1982.
[7] J. R. Dillon, A. Nasim, and E. R. Nestmann, eds., "Recombinant DNA Methodology," p. 13. Wiley, New York, 1985.
[8] R. DeWachter and W. Fiers, this series, Vol. 21, p. 167.

*Denaturing Running Buffer*

Urea gels: 1× TBE, pH 8.3 (see above)
Formamide gels (1×):

| | |
|---|---|
| 0.016 *M* Disodium hydrogen phosphate ($Na_2HPO_4$) | 6.8 g |
| 0.004 *M* Sodium dihydrogen phosphate ($NaH_2PO_4$) | 1.6 g |
| Water to | 3 liters |
| pH 7.5 | |

*Preparation of Polyacrylamide Gels*

*Caution: Acrylamide monomer is highly toxic and readily absorbed through the skin.* A mask and gloves should be worn for handling the solid and gloves for handling the solutions. The polymerized material is considered nontoxic.

*Concentrated Stock Solutions*

| | |
|---|---|
| 30% Acrylamide (29.1 acrylamide : bis) | 100 ml |
| Acrylamide | 29 g |
| *N,N'*-Methylenebisacrylamide | 1 g |
| Water to | 100 ml |
| Filter | |
| Ammonium persulfate, 10% freshly prepared | |

1. Clean glass plates are rinsed with ethanol and set aside to dry. A variety of plates are commonly used, depending on the design of the electrophoresis apparatus. A general protocol for assembling the plates involves placing the side spacers (and an optional bottom spacer) on the horizontal outer plate, lowering the second plate into position, and clamping and/or taping the sides and bottom of the plates with electrical tape to make a good seal.

2a. *Nondenaturing gels.* The stock acrylamide solution is mixed with water and 10× TBE to yield the appropriate percentage, volume, and buffer concentration (1×), filtered, and gently deaerated by swirling (dissolved oxygen will retard polymerization).

2b. *Denaturing gels containing 7 M urea.* Ultrapure-grade urea (42 g per 100 ml final volume) is added to appropriate volumes of stock acrylamide solution and 10× TBE, and dissolved by stirring, addition of water, and gentle warming (37–42°). After the urea has dissolved, the volume is adjusted with water and the mixture cooled to room temperature. The mixture may be filtered and deaerated if desired.

3. For either of the above types of gel add 50 μl (per 100 ml acrylamide solution) of *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 1 ml (per 100 ml) 10% ammonium persulfate, the polymerization initiator and catalyst respectively, and mix well.

4. Pour the gel by tilting the plates backward. Fill almost to the top and insert the comb immediately, leaving sufficient room between the top of the glass plate and the top of the teeth to remove the comb after polymerization. Clamping the comb in place results in wells free of thin polyacrylamide films on the walls and allowing the gel to polymerize in a near-horizontal position assures uniform thickness (important for thinner gels) and decreases the chance of leakage. The gel can be "topped up" if necessary to compensate for shrinkage during setting and *slow* leakages. Polymerization times vary with acrylamide percentage; 20% gels will set in approximately 30 min while lower percentage gels take about an hour at room temperature. A useful guide is to watch the unused gel solution, remembering that it will always remain liquid on the surface where air inhibits polymerization.

5. After removing the comb, the wells are rinsed with water, the bottom spacer or tape removed, and the gel attached to the electrophoresis apparatus. The reservoirs are filled with 1× buffer, air bubbles below the bottom of the gel removed with a bent needle and syringe, and the wells flushed with electrophoresis buffer using a Pasteur pipet. It is important to rinse the wells immediately prior to loading, especially for urea-containing gels. It is customary to prerun polyacrylamide gels for a short time (15 min) to equilibrate gel buffer and running buffer and, in the case of thin denaturing gels, to reach running temperature.

The procedure for polyacrylamide gels containing formamide is somewhat different. The following protocol is for 75 ml final gel solution.

1. 100 ml formamide (99%) is deionized within 1–2 days of use with 5 g mixed-bed resin (Bio-Rad AG501-X8, 20–50 mesh) by stirring for 1 hr. After filtration, deionized formamide is stored at −20°.

2. Acrylamide and bis (amounts in Table II) are dissolved in a final volume of 74 ml deionized formamide. Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$, 0.32 g), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot$

TABLE II
ACRYLAMIDE GEL CONCENTRATIONS FOR USE WITH FORMAMIDE

| Gel (%) | Acrylamide (g) | *N,N'*-Methylenebis-acrylamide (g) |
|---|---|---|
| 4 | 2.55 | 0.45 |
| 5 | 3.29 | 0.56 |
| 6 | 3.82 | 0.68 |
| 7 | 4.48 | 0.79 |
| 10 | 6.38 | 1.13 |
| 15 | 9.55 | 1.70 |

$H_2O$, 0.04 g), and ammonium persulfate (0.10 g) are dissolved in 1 ml water, added to the formamide solution, and filtered. Polymerization is initiated as usual with TEMED and the gel left overnight before use. Formamide gels are not prerun.

*Sample Preparation*

Generally speaking, DNA samples may be loaded onto nondenaturing polyacrylamide gels simply by adding a concentrated Ficoll solution (to increase sample density) containing marker dyes to assist in sizing. Restriction enzyme digests are most conveniently terminated by adding 0.1 volume 0.5 *M* EDTA and can then be loaded in Ficoll solution without further workup. Ficoll is preferred over other compounds (e.g., sucrose or glycerol) because it minimizes trailing at the sample edges which could lead to contamination in close-running preparative samples.

The preferred 10× loading solution for nondenaturing polyacrylamide gels is

30% Ficoll
0.25% Bromphenol blue
0.25% Xylene cyanole FF
0.20 *M* EDTA, pH 8
in 10× TBE (see above; nondenaturing running buffer)

For DNA preparations in low-ionic-strength buffers (e.g., restriction enzyme buffers) and at concentrations less than 1 $\mu$g/$\mu$l, 0.1 volume of the 10× loading solution is added prior to loading. For some DNA samples, in particular λ DNA, sticky single-stranded ends should be dissociated immediately prior to loading. This is best done by heating 5 min at 65°, followed by rapid cooling at 0°.

In certain cases, most commonly when the sample is too dilute or too high in salt or contains large amounts of protein or other impurities, it is advisable to improve the sample by a combination of phenol extraction and ethanol precipitation (see this volume [4,5]). A convenient way to concentrate a volume of dilute DNA too large to load directly is as follows.

1. Add 2–3 volumes of 2-butanol (*sec*-butyl alcohol) to the sample, mix, and keep the *lower* aqueous phase. Repeat as necessary until the lower phase is at an appropriate volume (typically 100–200 $\mu$l).
2. Extract the aqueous phase with 1 volume of chloroform. Keep the *upper* aqueous phase, which contains the DNA. Salt is also concentrated by this procedure.
3. Ethanol precipitate according to the standard protocol.

RNA is invariably phenol extracted and ethanol precipitated at some stage in a protocol prior to loading on a gel. It can then be treated as above. Nucleic acid samples for denaturing polyacrylamide gels are treated as follows. It is advisable, prior to loading, to remove salt from the samples (salt stabilizes duplex DNA). This is done by ethanol precipitation followed by rinsing in 70% ethanol–water.

1. Dissolve the pellet in an appropriate volume of deionized formamide.
2. Add 0.1 volume of 10× marker dyes (0.25% bromphenol blue, 0.25% xylene cyanole FF).
3. Heat 2 min at 100° and load.

*Gel Loading*

The techniques of loading gels are largely a matter of personal prejudice and whichever method allows a slow, steady delivery of sample should be used. Gels are always loaded with both reservoirs filled with buffer and the power turned off. Thin gels (less than 0.5 mm) are loaded with hand-drawn capillaries (see chapters on DNA sequencing [56–59]). Thicker gels may be loaded with glass capillaries attached to a mouth tube or hand dispenser or with an automatic pipettor according to convenience. The wells should be flushed just before loading and ethanol traces should be removed from the sample to ensure that it sinks.

*Size Markers for DNA Gels*

Dyes such as bromphenol blue and xylene cyanole FF are routinely used in gel electrophoresis, not so much as size markers but as indicators of how far a gel should be run to obtain the required result. For a more accurate size estimation it is most convenient to run a suitable restriction digest of commonly available DNA alongside the experimental samples. Accurate restriction maps based upon complete sequence data are available for plasmid pBR322 and λ*c*Its857 for example, and a judicious choice of enzyme will generate a simple restriction pattern with molecules spanning the desired size range. Manufacturers' catalogs frequently tabulate this data for easy reference. For example, λ DNA cut with *Hin*dIII and φx174 replicative form DNA cut with *Hae*III make a convenient set of markers from 70 bp to 20 kbp. The products of restriction enzyme digests can readily be isotopically labeled (see this volume [10]) to provide markers for use with labeled samples. It is frequently difficult to obtain multiple size markers in the lower size ranges by restriction digestion but the availability of synthetic DNA, ideal for use as size markers on denaturing

polyacrylamide gels, has overcome this problem. It is convenient to keep frozen samples of various markers in loading solution (100 ng/$\mu$l) ready for immediate use.

*Size Markers for RNA Gels*

Because of the structural complexity of single-stranded RNA and the substantial changes in relative mobility known to occur as a function of temperature, ionic strength etc., molecular weight determinations of nondenatured RNA by comparative methods are extremely unreliable. Consequently, when RNA is run with size markers, it is always done so under denaturing conditions. A number of small RNA markers (e.g., tRNAs, 5 S rRNA, 5.8 S rRNA) are appropriate and several well-characterized mRNAs ($\alpha$- and $\beta$-globin) can be used for larger markers on polyacrylamide. Glyoxylation of nucleic acids prior to electrophoresis renders DNA and RNA electrophoretically equivalent for gel electrophoresis and allows glyoxylated DNA restriction fragments of known size to be used as calibration markers for glyoxylated RNA samples.

*Electrophoresis Conditions*

Polyacrylamide gels are run at room temperature without cooling for most purposes. Generally speaking, large duplex DNA fragments are best resolved at low-voltage gradients (8 V/cm or less) whereas small fragments are run faster to limit diffusion. Denaturing gels are commonly run warm to limit duplex formation. A balance between the considerations of fragment length, resolution required, and available time is often determined empirically by each experimenter in each situation.

*Detection of Nucleic Acids in Polyacrylamide Gels*

After the electrophoresis run is completed, running buffer is removed from the reservoir and the gel plates detached from the apparatus. After removing any remaining tape and with the gel and plates lying flat, the upper plate is gently pried off from one corner (a spatula is most useful) leaving the gel on the lower plate. The spacers are removed. Several options arise at this point depending on the size, thickness, and percentage of the gel and how the nucleic acid is to be detected. Nonradioactive DNA and RNA are most frequently visualized after staining with the intercalating dye ethidium bromide.[9] As little as 10 ng duplex DNA per band is visible in a polyacrylamide gel. The following protocol is the

[9] C. Aaij and P. Borst, *Biochim. Biophys. Acta* **269**, 192 (1972).

method of choice for staining polyacrylamide gels. *Caution:* Ethidium bromide is mutagenic and gloves should be worn when handling even dilute solutions. It is most conveniently stored as a 5 mg/ml solution in water in a dark bottle.

1. Immerse the gel and supporting glass plate in a solution of 1× TBE containing 0.5 μg/ml ethidium bromide. Staining is carried out for at least 30 min. The solution should just cover the gel.
2. Wearing gloves, remove the gel with care using either the glass plate or a used piece of X-ray film as a support and transfer it onto a piece of plastic wrap (Saran Wrap) on a viewing and photographing surface. In some instances, particularly when it is desirable to reduce background fluorescence, the gel is destained by immersion in water for 15 min prior to viewing.

Ethidium bromide can also be used to detect single-stranded DNA and RNA but the nucleic acid–dye complex is weak and the fluorescent yield low resulting in 5- to 10-fold lower, but still adequate, sensitivity for most purposes. Several other dyes have been used to stain nucleic acids in gels.[10,11] Silver staining[12] has been reported to be more sensitive than ethidium bromide staining and may be preferable when high sensitivity is needed and for denaturing gels.

Methylene blue is an alternative dye for use with preparative samples and avoids the hazards of UV detection. The gel is stained in 0.02% methylene blue, 10 m*M* Tris–acetate (pH 8.3) for 1–2 hr at 4°. Avoiding direct sunlight, the excess stain is washed out with several changes of water (5–8 hr) and the bands become visible. The limit of detection is about 250 ng/1 cm band.

A common method for detection of synthetic oligonucleotides in denaturing polyacrylamide gels is that of UV shadowing. The gel is removed from the glass plates and placed on a fluorescent chromatography plate (silica gel 60-F254, E. Merck) covered in plastic wrap. A short-wave hand-held UV monitor is used to locate the DNA which appears as a black shadow on a green background and can readily be excised. More than 1 μg DNA per band can be detected by this method. Exposure to the light should be as short as possible. Because it is a shadowing technique,

[10] O. Gaal, G. A. Medgyesi, and L. Vereczkey, "Electrophoresis in the Separation of Biological Molecules." Wiley, New York, 1980.
[11] A. T. Andrews, "Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications." Oxford Univ. Press (Clarendon), London and New York, 1981.
[12] C. R. Merril, R. C. Switzer, and M. L. Van Keuren, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4335 (1979).

when excising the DNA the light should be vertically above the band to avoid cutting errors.

*Viewing and Photography*

*Caution:* Safety glasses and preferably a face mask should be worn around UV light sources.

The ethidium bromide–nucleic acid complex absorbs UV irradiation at 260 nm (the nucleic acid absorption maximum) or 300 nm (the bound ethidium maximum). The fluorescence of ethidium stacking in duplex DNA is 10 times greater than that of free ethidium bromide and the emission maximum is at 590 nm (red orange). The wavelengths of commercially available UV sources (254, 302, and 366 nm) have been compared for efficiency and deleterious nicking and dimerization of the DNA.[13] The two sources at 254 and 302 nm give the greatest fluorescence, but damage to DNA at 254 nm is extensive and a 302-nm source is to be preferred, especially for preparative work. Unfortunately, these lamps must be used with a filter to eliminate a red light emission which cuts down DNA sensitivity for detection of faint bands. In addition, nicking DNA by illumination at 254 nm may be advantageous if the DNA is large and ultimately will be transferred to membranes for hybridization. Ethidium complexes may be viewed by illumination either from the sides (incident light) with the gel on a black surface or from below (transmitted light). The transillumination system is the method of choice, being both more sensitive and requiring much shorter exposure times. Commercially available UV transilluminators are supplied with a UV-pass, visible blocking filter (an excitation filter). A Polaroid MP4 camera with a Kodak Wratten 22A or 23A red filter (to remove the red emission from the UV lamp) is positioned above the gel. Type 52 (positive) or 55 (positive and negative) film is used with a 545 film holder. Exposure time is typically about 5 sec. Faint bands are often detected by developing the negative.

*Autoradiography of Gels*

This subject has been covered in more detail elsewhere in this volume [7] and will only be briefly summarized here. Thick polyacrylamide gels (>1 mm) are generally removed from both glass plates, wrapped in plastic wrap, and exposed to X-ray film. Alignment of gel and film is aided by labels prepared with "radioactive ink" (commonly, old $^{32}P$ added to ink).

[13] C. F. Brink and L. Simpson, *Anal. Biochem.* **82,** 455 (1977).

Thin gels or large and unwieldy thick gels are either left on the glass plate, covered with plastic wrap, and exposed to film (the glass plate should be above the gel and film in the cassette to aid good contact) or, for thin gels, peeled off onto a suitable backing material (old X-ray film is best if gel slices are to be excised) and exposed to film.

*Sizing and Quantitation of Nucleic Acids in Polyacrylamide Gels*

As described previously, and because to a first approximation mobility is independent of sequence or composition, DNA and RNA fragment sizes are estimated from their mobility relative to standards of known size. For most purposes, it is sufficient to obtain an estimate of the fragment size as follows. (1) Photograph the gel containing markers and samples along with a UV-transparent ruler. (2) Construct a standard graph for the markers by plotting mobility (measured in centimeters from the well base) versus the log of molecular weight or size. This is most conveniently plotted on semilog paper for polyacrylamide gels; the size range of fragments is such that this will yield a straight line. (3) The size of the sample fragments can be read directly from the graph using the measured mobilities.

Accurate estimation of sizes is helped, particularly for duplex DNA, by running the gel at low-voltage gradients. A more detailed discussion of sizing of nucleic acids utilizing glyoxal to denature prior to electrophoresis has been presented in this series.[14]

Accurate quantitation of DNA *in situ* in a gel is, for most purposes, not necessary in the context of molecular cloning. Many experimenters are content to estimate by direct visual comparison with known quantities and obtain a feel for what 100 ng of duplex DNA looks like, for example, in an ethidium-stained gel. A sophisticated method involving microdensitometer tracing and comparison to known standards has been previously described.[15] The gel is stained with ethidium bromide and photographed under UV illumination. Microdensitometer tracing of the photographic negative yields pen deflections which are related to the amount of DNA in the band. The absolute quantities are determined by constructing a standard curve for the film, relating pen deflection, exposure, and optical density and using DNA standards of known size. It is more customary and useful for cloning purposes to quantitate nucleic acids after elution from a gel by standard spectrophotometric methods (see this volume [6]).

[14] G. C. Carmichael and G. K. McMaster, this series, Vol. 65, p. 380.
[15] A. Prunell, this series, Vol. 65, p. 353.

*Elution from Polyacrylamide Gels*

A detailed overview of the recovery of DNA from gels of all kinds has been previously presented in this series.[16] This section will provide a brief summary. The two primary concerns in selecting gel elution techniques are the percentage recovery of the nucleic acid and the nature of contamination which may adversely affect subsequent steps in the protocol. For polyacrylamide gels the most common and reliable elution technique, applicable to both RNA and DNA, is that of crush elution in high-ionic-strength buffer.[17,18] Recovery of nucleic acids (to ~1 kb in length) is almost quantitative especially when thin gels (0.5 mm) are used. The major impurity remaining through cleanup procedures is linear acrylamide, which does not inhibit most enzymes (restriction enzymes, ligase, polymerase) with the exception of reverse transcriptase. If mRNA is fractionated electrophoretically on polyacrylamide gels it will be necessary to repurify by oligo(dT)-cellulose chromatography. A more appropriate protocol for mRNA is presented later. For RNA work, solutions must be sterile and all glassware acid washed. It is also advisable to include 0.1% sodium dodecyl sulfate (SDS) and 1 m*M* EDTA to impair ribonucleases. A general protocol is as follows.

1. Excise the desired bands from the gel with a razor blade and forceps. For nonradioactive samples, keep exposure to UV light at a minimum.
2. For denaturing gels only, it is advisable to soak the gel slice in water for 15 min to remove urea, formamide, and phosphate buffer. It is particularly important to remove phosphate to prevent a large coprecipitate with gel impurities which can trap nucleic acids. Small oligonucleotides (<30) will begin to elute in water.
3. The gel slice is transferred to a 1.5-ml polypropylene tube, siliconized scintillation vial, or Corex tube depending on its size and is crushed by one of several methods. (This is not necessary for thin gels—one of their advantages—or for small nucleic acids, e.g., synthetic oligonucleotides). An acid-washed spatula can be used to pulverize the gel in the scintillation vial or Corex tube. An efficient way to crush gels is to extrude them from a syringe barrel through a shortened wide-gauge needle (broken with pliers) into a polypropylene tube by low-speed centrifugation (5 min) in a swinging bucket rotor.

[16] H. O. Smith, this series, Vol. 65, p. 371.
[17] W. Gilbert and A. Maxam, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3581 (1973).
[18] R. Yang, J. Lis, and R. Wu, this series, Vol. 68, p. 176.

4. Add gel elution buffer. The volume depends on the size of gel slice but as a guide 0.5–1 ml is used for a slice 1 cm × 1.5 mm. Gel elution buffer: 0.5 *M* ammonium acetate, 10 m*M* magnesium acetate, 1 m*M* EDTA, 0.1% SDS. Incubate overnight at 37°. Agitation and higher temperature (60°) may be used for larger molecules and larger gel volumes.

5. Fragments of polyacrylamide are removed by centrifugation and filtration. Quik-Sep columns (Isolab) work well for large volumes as they fit 15-ml Corex tubes. A Pasteur pipet plugged with siliconized glass wool can be used for smaller volumes.

6. Ethidium bromide can be removed by extraction with 2-propanol or isobutanol saturated with elution buffer and volumes can be reduced, prior to precipitation, with 2-butanol as described previously. Alternatively, DNA is adjusted to 0.8 *M* LiCl, 50 m*M* Tris (pH 9.0) and extracted with an equal volume of phenol–chloroform.

7. Ethanol precipitate and wash with 70% ethanol–water according to standard procedures. This step is not necessary, for example, if the nucleic acid is radioisotopically labeled and is to be used directly for hybridization.

Repeated elution will serve to increase the yield if necessary.

Nucleic acids may also be recovered in high yield from polyacrylamide gels by electroelution. The technique is described in the following section, dealing with agarose gels, where it is more commonly used.

A number of commercially available resins have been introduced in recent years for post-elution purification of nucleic acids. As with DEAE-cellulose, nucleic acids bind to minicolumns of the material in low salt, allowing impurities to pass through by washing and can be eluted in higher salt. Two such materials are NACS-52 (Bethesda Research Laboratories) and Elutip-d (Schleicher and Schuell). Comprehensive applications manuals are provided by the suppliers. A technical description of the properties of a similar resin (RPC-5) has been presented previously in this series.[19,20]

### Agarose Gel Electrophoresis

The resolving power of agarose gels is a function of the concentration of dissolved agarose. The migration rate of nucleic acids through agarose gels is additionally dependent upon the molecular size (for linear frag-

[19] R. D. Wells *et al.*, this series, Vol. 65, p. 327.
[20] J. A. Thompson *et al.*, this series, Vol. 100, p. 368.

ments), conformation, and voltage gradient. Under certain conditions, the resolution on the basis of conformation (circular, nicked circle, or linear), is a particularly useful property. The effective range of separation for agarose gels of various percentages is shown in Table III.

Denaturants commonly added to agarose gels for analysis of single-stranded nucleic acids are sodium hydroxide (DNA only), formaldehyde, and methylmercuric hydroxide. The latter is extremely toxic and results in large volumes of hazardous buffer. This disadvantage should be weighed against its undisputed ability to denature nucleic acids completely. In many instances, glyoxylation of DNA or RNA prior to electrophoresis can be effectively substituted. Typical applications of agarose gels are analysis of restriction enzyme digests of cloned DNA, preparation of cut vectors and fragments for cloning, sizing large DNA and RNA, $S_1$ mapping, and analysis of cDNA cloning intermediates.

*Equipment*

The horizontal position has emerged in recent years as the preferred position for agarose gel electrophoresis. It has the advantages of simplicity in pouring, loading, and handling, versatility in size using the same apparatus, and support from below, important for low percentage gels. Tanks are available in all sizes commercially including those for minigels and can be cheaply made according to a standard design (ref. 1, p. 42; ref. 6, pp. 154–155, 163). The essence of the design is that the gel is poured on a glass plate with the comb in place. When set, it is transferred on the plate to a platform and is submerged in running buffer. It is common to use a microscope slide if only three or four lanes are required. The gel is poured relying on surface tension and the comb held in place with a clamp.

TABLE III
RANGE OF SEPARATION FOR AGAROSE GELS

| Agarose (%) | Optimal range of separation linear DNA (kb) |
|---|---|
| 0.3 | 60–5.0 |
| 0.6 | 20–1.0 |
| 0.7 | 10–0.8 |
| 0.9 | 7–0.5 |
| 1.2 | 6–0.4 |
| 1.5 | 4–0.2 |
| 2.0 | 3–0.1 |

*Nondenaturing Agarose Gels*

Agarose powder comes in many grades. For general analytical electrophoresis purposes the best agarose is type II low endo-osmotic agarose. This type, however, contains contaminants which coelute with DNA and inhibit most commonly used enzymes, which means that DNA must be extensively purified following elution from this kind of gel. An extremely attractive alternative for preparative cloning work involves the use of high-quality, low melting temperature agarose. This agarose melts at 65 and sets at 30°, which allows DNA to remain double-stranded and also allows many enzymes to be used in the liquid agar. T4 DNA ligase reportedly functions efficiently in the solidified gel at 15°. A collection of methods has been recently compiled by Struhl[21] involving low melting temperature agarose gel electrophoresis and subsequent "in-gel" manipulation of fragments including transformation. The source of agarose for this application appears critical and Sea Plaque low melting temperature agarose (Marine Colloids) is recommended. Horizontal agarose gels are generally about 3 mm thick (thinner gels should be run in the vertical position), and are prepared as follows.

1. Powdered agarose is added to the desired electrophoresis buffer (1×) to give the correct percentage. It is common to make 100 ml of gel solution. A typical minigel apparatus will require 25 ml. The agarose is dissolved completely by heating, most conveniently in a microwave oven. Care should be taken to ensure that the solution is homogeneous.
2. The solution is allowed to cool to approximately 50–60° before pouring. Agarose above this temperature can deform some gel trays and combs. Cooling the agarose is also important where surface tension is being used to contain the liquid gel. At this stage, there is the option of adding ethidium bromide (0.5 μg/ml, final) to the gel.[22] This allows the progress of the gel run to be monitored during the run by incident or transmitted longwave UV light (many horizontal gel apparatuses are made of UV-transparent Plexiglas). Incorporation of ethidium will, however, affect the conformation of certain DNA molecules, thereby changing their mobility. To minimize the volume of dilute ethidium bromide and to minimize damage to the DNA ethidium complex by light, it is recommended that gels be run without inclusion of ethidium bromide.
3. Pour the gel into the mold. The precise nature of this operation will depend on the design of the apparatus. In some, tape is used to complete

[21] K. Struhl, *BioTechniques* **3,** 452 (1985).
[22] R. A. Sharp, B. Sugder, and J. Sambrook, *Biochemistry* **12,** 3055 (1973).

the mold in the apparatus itself (e.g., Bethesda Research Laboratory); in others, the gel is poured in a mold separate from the apparatus (e.g., International Biotechnologies Inc.). The comb is generally in place when the gel is poured. It is important that the bottom of the comb does not touch the plate underneath the gel.

4. After setting (~30 min; the gel should look uniformly opalescent), any tape is removed, the gel submerged in buffer, and the comb gently removed. The apparatus is topped up with buffer until the gel surface is just submerged. Ethidium-containing gels may be run in ethidium-containing buffer. Agarose gels are not prerun.

*Nondenaturing Running Buffers*

Several running buffers are in common use for nondenaturing agarose gel electrophoresis. Tris–borate (see section on polyacrylamide gels) is often used, at 0.5× concentration, but interacts with agarose and leads to low recovery of nucleic acids in preparative gels. Tris–acetate buffer[23] is commonly used and is ideal for preparative work. The buffering capacity is low and so recirculation of buffer on long runs is advisable. Tris–phosphate has good buffering capacity, obviating the need for circulation, but should be avoided if the fractionated sample is to be eluted and ethanol precipitated because of coprecipitation of phosphate. The buffers are stored as concentrated stocks and diluted as needed.

| | |
|---|---|
| 50× Tris–acetate (TAE): | |
| Tris base | 242 g |
| Glacial acetic acid | 57.1 ml |
| 0.5 *M* EDTA (pH 8) | 100 ml |
| Water to | 1 liter |
| TAE working solution: | |
| Tris–acetate | 0.04 *M* |
| EDTA | 0.001 *M* |
| 10× Tris–phosphate: | |
| Tris base | 108 g |
| 85% Phosphoric acid | 15.5 ml |
| 0.5 *M* EDTA (pH 8) | 40 ml |
| Water to | 1 liter |
| Tris–phosphate working solution: | |
| Tris–phosphate | 0.08 *M* |
| EDTA | 0.002 *M* |

[23] V. E. Loening, *Biochem. J.* **102,** 251 (1967).

*Sample Preparation and Loading*

As with polyacrylamide gel electrophoresis, samples for nondenaturing agarose gels are conveniently loaded by addition of a concentrated Ficoll, dye, EDTA solution (see previous section). The capacity of a standard well (~2 mm deep × 5 mm wide) is about 200 ng per band, and as little as 1–5 ng of double-stranded DNA may be detected by ethidium bromide staining in agarose. Typically, restriction analysis of a recombinant plasmid or phage will involve running 0.5–1 μg DNA per lane. For restriction analysis of genomic DNA where many bands will run such as to produce a smear, 5–10 μg of DNA per lane can be loaded.

*Alkaline Agarose Gels*[24]

Denaturing agarose gels containing sodium hydroxide are most useful for single-stranded DNA analysis and are frequently used analytically for checking first- and second-strand synthesis in cDNA cloning and the size range of nick-translated DNA (see elsewhere in this volume). Agarose is hydrolyzed at high temperature by alkali and the gels are best made by preparing the gel as in the previous section, but in a neutral buffer (50 m*M* NaCl, 1 m*M* EDTA). When set, the gel is soaked by submersion in alkaline running buffer (30 m*M* NaOH, 1 m*M* EDTA) for at least 30 min before running.

DNA samples should be phenol extracted and ethanol precipitated and the dried pellet dissolved in

Loading buffer:

| | |
|---|---|
| 50 m*M* | Sodium hydroxide |
| 1 m*M* | EDTA |
| 3% | Ficoll |
| 0.025% | Bromocresol green |
| 0.025% | Xylene cyanole FF |

The gel, which has been equilibrating in alkaline running buffer for at least 30 min, is drained of excess buffer (leave ~1 mm covering the gel) prior to loading.

*Glyoxal Denaturation of Nucleic Acids*[14]

Glyoxal denaturation of RNA and of DNA prior to electrophoresis allows molecules to remain denatured throughout electrophoresis, resulting in accurate separation by molecular weight and quantitative transfer to filters for Northern blot analysis. Ease of handling and lack of toxicity

[24] M. W. McDonell, M. N. Simon, and F. W. Studier, *J. Mol. Biol.* **110**, 119 (1977).

recommend it over the other commonly used method of formaldehyde denaturation. As these two methods cover the same needs, we recommend the use of glyoxal–DMSO denaturation for electrophoresis. However, greater sensitivity has been obtained in Northern blot experiments (this volume [61]) using formaldehyde gels.

Glyoxal (6 *M*, 40% solution) should be deionized with a mixed-bed ion-exchange resin such as Bio-Rad AG501-X8 until neutral, then distributed in useful aliquots into tightly capped tubes, and maintained at −20°. Glyoxal should not be reused after opening.

To denature, mix

1 *M* Glyoxal
50% (v/v) Dimethyl sulfoxide
10 m*M* Sodium phosphate buffer, pH 7.0
DNA or RNA (up to 10 μg/8 μl reaction volume)

Incubate at 50° for 1 hr. Cool in ice.

Add 2 μl loading buffer/8 μl reaction.

Loading buffer:

10 m*M* Sodium phosphate (pH 7.0)
50 % (v/v) Glycerol
0.4 % Bromphenol blue

Gel: 0.75–1.5% agarose in 10 m*M* sodium phosphate (pH 7.0). As markers, use similarly glyoxylated RNAs or DNAs. Run the gel at 3–4 V/cm for 10 hr, with constant recirculation of buffer to maintain pH at 7.0. Glyoxal readily dissociates from DNA and RNA at pH 8.[25]

Nucleic acids can be visualized following electrophoresis by staining with ethidium bromide (0.5 μg/ml in running buffer) and photographed as described above. If the RNA is to be transferred to a filter for hybridization analysis it is recommended that the gel not be stained, since any soaking of the gel prior to transfer has been shown to reduce transfer.[25]

Staining with acridine orange is a more sensitive method to visualize glyoxylated DNA and RNA.[14] Agarose gels are stained for 30 min and polyacrylamide–agarose composite gels for 15 min in 30 μg acridine orange/ml in 10 m*M* sodium phosphate (pH 7.0). Destaining is accomplished by running hot tap water over the gel for 5–10 min. Stained molecules are then visualized by UV illumination (254 nm). After destaining, double-stranded nucleic acids appear green and single-stranded nucleic acids (i.e., most RNAs and denatured DNA) appear orange. Color photographs using Polaroid 108 color film and a yellow filter can be taken, or black and white photographs using Polaroid 105 positive/negative or 107C positive film and a red filter.

[25] P. S. Thomas, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5201 (1980).

*Methylmercuric Hydroxide Gels*[26]

Methylmercuric hydroxide gels are used for accurate sizing of DNA and RNA and for electrophoresis of RNA prior to transfer to membranes for hybridization. These gels are particularly useful when fractionated mRNA is to be eluted and used for *in vitro* translation studies. The risks involved in working with methylmercuric hydroxide should be fully understood and precautions against its potentially lethal effects must be taken. This information has been published previously.[27] *It is poisonous and somewhat volatile and all manipulations must be carried out in a fume hood.* It is strongly recommended that alternative methods (e.g., glyoxylation prior to electrophoresis for molecular weight determination or transfer to membranes for hybridizations) be tried first. Agarose gels containing methylmercuric hydroxide are made as follows.

1. The desired amount of agarose is dissolved in running buffer (see below for methylmercuric hydroxide gel running buffer) by heating.
2. After cooling to 50–60°, add methylmercuric hydroxide to 5 m*M* final concentration and pour the gel. It is not necessary to include methylmercuric hydroxide in the running buffer.

Denaturation with methylmercuric hydroxide is reversible and nucleic acids can be eluted after soaking the gel in 0.5 *M* ammonium acetate, 10 m*M* dithiothreitol, or 2-mercaptoethanol for 1 hr.

The buffer should be recirculated during the run.

Running buffer (1×):

0.05 *M* Boric acid
0.005 *M* Sodium borate
0.010 *M* Sodium sulfate
pH 8.2

Equal volumes of RNA (10 μg can be loaded per 0.5 cm lane) and 2× loading buffer are mixed.

Loading buffer (2×):

| | |
|---|---|
| 1 *M* Methyl mercuric hydroxide | 25 μl |
| 4× Running buffer | 500 μl |
| 30% Ficoll | 200 μl |
| Water to | 1 ml |
| Bromphenol blue | 0.1% |
| Xylene cyanole FF | 0.1% |

Use of ethidium bromide in RNA gels should be avoided if the RNA is to be transferred to membranes for hybridization. Waste solutions and gels containing methylmercuric hydroxide should be treated before dis-

[26] J. M. Bailey and N. Davidson, *Anal. Biochem.* **70,** 75 (1976).
[27] J. E. Cummins and B. E. Nesbitt, *Nature (London)* **273,** 96 (1978).

posal with excess of a sulfhydryl reagent such as dithiothreitol or 2-mercaptoethanol. For the gel, treatment with 10 m*M* dithiothreitol for 1 hr is sufficient.

*Formaldehyde Gels*[28]

*Caution:* Concentrated formaldehyde solutions should be stored and used in a fume hood.

Formaldehyde–agarose gels are used for fractionation of RNA prior to transfer to hybridization membranes. They are prepared as follows. Formaldehyde (37% in water, 12.3 *M*) and 5× running buffer (see below) are added to the desired percentage of molten agarose in water (50–60°) to give final concentrations of 1× buffer and 2.2 *M* formaldehyde.

Running Buffer (1×):

0.04 *M* Morpholinopropanesulfonic acid (MOPS), pH 7.0
0.01 *M* Sodium acetate
0.001 *M* EDTA

RNA samples (up to 20 μg in 5 μl) are incubated at 55° for 15 min or 65° for 5 min in:

| | |
|---|---|
| 5× gel running buffer | 2 μl |
| Freshly deionized formamide | 10 μl |
| Concentrated formaldehyde | 3.5 μl |

2 μl of the sterile loading buffer is added.

Loading buffer:

30 % Ficoll
1 m*M* EDTA
0.25 % Bromphenol blue
0.25 % Xylene cyanole FF

*Size Markers*

As discussed previously, coelectrophoresis of fragments of known size provides the best method for estimating the size or molecular weight of a sample. The essential condition is to choose a gel system for which the log molecular weight is linearly related to mobility. The useful size range of agarose gels requires the availability of larger known size markers, and frequently restriction digests of λ DNA provide the necessary fragments to construct a standard straight line. A mixed *Eco*RI/*Hin*dIII digest or *Bgl*II digest is frequently used for size markers on agarose for larger fragment separations.

Agarose gels are frequently used to determine the molecular conformation of plasmid DNA: circular, nicked, linear, multimeric, etc. The relative running order and mobilities of the conformers depend on the

[28] H. Lehrach, D. Diamond, J. M. Wozney, and H. Boedtker, *Biochemistry* **16**, 4743 (1977).

buffer, current, and agarose concentration and known circular or linear markers in the appropriate size range should be used. Such gels are frequently run in the presence of ethidium bromide, which (usually at 0.5 μg/ml) increases the mobility of form I (closed circular) DNA and retards that of nicked (form II) and linear (form III) molecules.

For denaturing gels, the most convenient size markers are fully denatured DNA restriction fragments or RNAs of defined size (e.g., several ribosomal RNAs). As a general rule, markers are treated identically to samples and run in whatever type of gel system is chosen. For formaldehyde gels, labeled markers or markers that will be labeled after hybridization are best. Formaldehyde-denatured nucleic acids are not well visualized by ethidium staining. Formaldehyde can be removed from isolated marker lanes by washing in water (4 × 30 min) and 0.1 *M* ammonium acetate (2 × 30 min) and staining for 1 hr in 0.1 *M* ammonium acetate, 0.1 *M* 2-mercaptoethanol with ethidium bromide (0.5 μg/ml). As mentioned earlier, glyoxylated DNA or RNA samples can be sized by using glyoxylated DNA restriction fragments which closely span the desired size range. This is the most convenient and least hazardous method for size determination.

### *Electrophoresis Conditions*

Agarose gels for most purposes are run at room temperature. Exceptions are low percentage agarose gels (<0.5%) which are easier to handle in the cold and low melting temperature agarose gels which may melt if run too fast at room temperature. The best results, in terms of resolution and accurate sizing, are obtained by running agarose gels very slowly (<5 V/cm). It is important to circulate or change buffer in such runs, especially for denaturing gels or glyoxylated samples, in order to avoid pH changes which could begin to reverse glyoxylation (pH >8).

### *Detection, Viewing, and Photography of Nucleic Acids in Agarose Gels*

The essentials of these techniques are identical to those used for polyacrylamide. Ethidium bromide is the dye of choice for both single- and double-stranded nucleic acids, the limits of detection in agarose being somewhat lower than in polyacrylamide (~1–5 ng per band for double-stranded DNA). The sensitivity for single-stranded nucleic acids is 5- to 10-fold less. As discussed above, in particular for looking at intact plasmids, ethidium bromide is frequently incorporated into gel and running buffer during electrophoresis. Otherwise gels are stained after electrophoresis in electrophoresis buffer containing 0.5 μg/ml ethidium bromide for 30 min.

*Determination of Fragment Size*

As with polyacrylamide gel electrophoresis, it is important to obtain a good linear relationship in the size range of interest between relative mobility and log size. The size of fragments on agarose can be large and errors in measurement potentially great. Errors are minimized by taking the following precautions: (1) Gels are run as slowly as is experimentally realistic. (2) Size markers are selected which closely bracket the unknown sample and thereby provide an accurate standard line. (3) In extreme cases, it is possible to scan photographs with a densitometer and obtain accurate relative mobilities by measuring peak separations. This method is seldom used in the course of routine molecular cloning.

*Elution from Agarose Gels*

A myriad of techniques exists for elution of nucleic acids from agarose gels—a direct consequence of no entirely satisfactory protocol being available. Reviews of the topic have been presented[16] and will be summarized and updated here. In addition to electroelution and diffusion which are also appropriate for polyacrylamide gels, nucleic acids can be eluted from agarose by gel dissolution and by physical extrusion. It is worth stressing at this point that for many purposes, use of high-quality low melting temperature agarose (discussed previously) eliminates the need for elution and the relatively high cost can be easily offset by frugal use and the time saved. The following methods are most commonly used for DNA.

*Electroelution Techniques*

*Into Dialysis Bags*[24]

1. The band of interest is excised with a razor blade and dropped into a dialysis bag tied off at one end and filled with elution buffer. A suitable elution buffer with low conductivity is 5 m*M* Tris, 2.5 m*M* acetic acid (pH 8).

2. After removing most of the buffer (the gel slice should be still surrounded) the bag is closed and immersed in elution buffer in an electrophoresis tank (a minigel apparatus works well for small slices). Current is passed through the bag (the gel should be aligned such that its smallest dimension is parallel to the electric field) for 2–3 hr at ~100 V.

3. The current is reversed for 5 min to remove the DNA from the inner walls of the dialysis bag.

4. Using a Pasteur pipet, remove the buffer from the bag. Elution of DNA from the gel slice can be monitored by restaining. The DNA can be

concentrated and further purified by absorption and elution from a minicolumn as described above.

*Into Troughs*

1. The ethidium bromide-stained DNA is localized using a longwave UV light. It is most convenient for this technique to have run the gel in a UV-transparent Plexiglas tray.

2. Cut a small trough directly in front of and 1–2 mm wider than the desired band, remove the buffer covering the gel, and continue electrophoresis. The DNA moves from the gel into the buffer, which is periodically removed and replaced with fresh until all the desired band has been recovered. DNA can be phenol extracted and ethanol precipitated (after removal of any agarose by filtration) and is usually free of inhibitors.

3. A time-saving modification[29] involves making a single incision directly below the band, inserting a piece of Whatman 3 MM paper and dialysis membrane (presoaked 5 min in buffer) into the incision with the paper adjacent to the band, and continuing electrophoresis. Constant monitoring is not required as the DNA sticks to the paper and membrane. The DNA is eluted from the paper with 3 × 100 $\mu$l 0.2 *M* NaCl, 50 m*M* Tris (pH 7.6), 1 m*M* EDTA. This is most conveniently achieved by placing the paper in a plastic pipet tip or punctured polypropylene tube, adding the buffer in 100-$\mu$l batches, and recovering the eluate by centrifugation into a polypropylene tube. The DNA is phenol–chloroform extracted and ethanol precipitated. Use of DEAE paper in place of 3 MM and a dialysis membrane is reported to eliminate the need for subsequent cleanup on DEAE, NACS-52, Elutip-d, or similar resins.[30]

*Physical Extrusion Techniques*

Commonly known as the freeze–squeeze procedure,[31] physical extrusion is ideal for large DNA recovery from low percentage (<0.6%) gels. Yields are normally >50%. The following technique works well.

The gel slice is placed between sheets of Parafilm or plastic wrap and frozen (30 sec on dry ice). As it defrosts, it is squashed between thumb and forefinger or against the lab bench, and the drop of liquid collecting on the plastic contains much of the DNA. The liquid is centrifuged to remove agarose particles and cleaned up as described elsewhere in this chapter. A number of modifications (reviewed in Smith[16]) may suit personal preferences.

[29] S. C. Girvitz, S. Bacchetti, A. J. Rainbow, and F. L. Graham, *Anal. Biochem.* **106,** 492.
[30] G. Dretzen, M. Bellard, P. Sassone-Corsi, and P. Chambon, *Anal. Biochem.* **112,** 295.
[31] R. W. J. Thuring, P. M. Sanders, and P. Borst, *Anal. Biochem.* **66,** 213 (1975).

*Isolation of DNA from Low Melting Temperature Agarose*

Not all protocols will allow "in-gel" manipulation of DNA in low melting temperature agarose. The following protocol is suitable for recovery of DNA from such gels.[32] The gel is run in 1× TAE.

1. To the excised gel slice add an equal volume of 0.1 *M* Tris–acetate (pH 7.5), 5 m*M* EDTA, 0.5 *M* NaCl and heat to 65° until the agarose has melted.
2. Extract with an equal volume of phenol. A white emulsion forms which separates on centrifugation. Powdered agarose appears at the interface.
3. The aqueous layer is reextracted with phenol–chloroform and chloroform, and the DNA recovered by ethanol precipitation.

RNA, most frequently mRNA, is often transferred from agarose gels to filters for hybridization. These techniques are covered in this volume [61]. The problem associated with mRNA isolation for, for example, translation studies is that standard procedures give poor recoveries and degraded mRNA. A method enabling "in-gel" translation of small (nanogram) quantities of fractionated poly(A)$^+$ mRNA suitable for rapid screening has been described.[33]

RNA may be recovered from low melting temperature denaturing gels (containing methylmercuric hydroxide) as follows.

1. The gel is soaked in 0.1 *M* dithiothreitol for 30 min and the excised slice dissolved in 4 volumes of 0.5 *M* ammonium acetate by heating of 65°.
2. Extract with an equal volume of phenol. Reextract with phenol–chloroform and chloroform to remove all agarose from the aqueous layer.
3. The extracted RNA is ethanol precipitated and is generally suitable for use with reverse transcriptase.

*Removal of Gel Contaminants*

As discussed previously, nucleic acids eluted from conventional agarose gels contain many inhibitors. A combination of phenol extraction and chromatography on DEAE-cellulose or a commercially available reverse-phase resin (discussed for polyacrylamide gels) is usually sufficient to remove contaminants. A typical procedure for DEAE-cellulose is as follows.

1. A small DE-52 (Whatman) column is equilibrated in 0.15 *M* NaCl, 50 m*M* Tris (pH 8), 1 m*M* EDTA, and DNA is loaded in the same or lower buffer concentration (the capacity is >200 μg/ml).

[32] L. Weislander, *Anal. Biochem.* **98,** 305 (1979).
[33] T. L. Brandt and P. B. Hackett, *Anal. Biochem.* **135,** 401 (1983).

2. The column is washed with 10 column volumes of loading buffer and the DNA eluted with 1 *M* NaCl, 50 m*M* Tris, 1 m*M* EDTA (1–2 column volumes).

## Gel Drying

Agarose and polyacrylamide gels can be dried by heating *in vacuo* using commercially available dryers. This is often performed prior to autoradiography (e.g., $^{35}$S-labeled dideoxy sequencing gels). For gels containing denaturants (e.g., urea) it is important to remove them before drying. This is most commonly achieved for urea by soaking for at least 15 min in 10% aqueous acetic acid. Agarose gels containing denaturants should also be washed prior to drying. *Never* dry methylmercuric hydroxide gels without ensuring complete removal of the toxic and volatile denaturant with 2-mercaptoethanol. Gels are placed on two sheets of Whatman 3 MM paper and covered with plastic wrap. For thin gels, the paper is evenly pressed onto the gel (after excess liquid from the 10% acetic acid fixing has been removed with Kimwipes) and the paper with gel attached is gently peeled off the glass plate. The gel is placed on the dryer and covered with a porous plastic sheet and a rubber flap.

Drying times vary with gel thickness and temperature. Sequencing gels typically take 30 min at 80°. Agarose gels should not be heated above their melting point! It is important not to break the vacuum before the gel is completely dry; this can be easily detected by turning off the heat and monitoring whether the gel feels cool (still drying). For autoradiography of $^{35}$S, the plastic wrap must be removed from the dried gel.

## Conclusion

The powerful techniques described in this chapter are constantly undergoing improvement and finding wider application. Two exciting areas of innovation which will find increasing application in molecular biology are the use of pulse-field gel elecrophoresis for resolution of very large duplex DNA molecules (e.g., chromosomes) (Smith and Cantor,[34] and references therein) and the use of gels to detect, map, and resolve point mutations within otherwise identical DNA molecules (Myers *et al.*,[35] and references therein). The access to hitherto intractable scientific problems afforded by these new applications of gel electrophoresis attest to its continuing central role in molecular cloning.

[34] C. L. Smith and C. R. Cantor, *Nature (London)* **319,** 701 (1986).

[35] R. M. Myers, S. G. Fischer, L. S. Lerman, and T. Maniatis, *Nucleic Acids Res.* **13,** 3131 (1985).