ELECTROPHORESIS THROUGH AGAROSE OR POLYACRYLAMIDE GELS lies near the heart of molecular cloning and is used to separate, identify, and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of fluorescent intercalating dyes, such as ethidium bromide or SYBR Gold; bands containing as little as 20 pg of double-stranded DNA can be detected by direct examination of the gel in UV. If necessary, these bands of DNA can be recovered from the gel and used for a variety of purposes.

Agarose and polyacrylamide gels can be poured in a variety of shapes, sizes, and porosities and can be run in a number of different configurations. The choices within these parameters depend primarily on the sizes of the fragments being separated. Polyacrylamide gels are most effective for separating small fragments of DNA (5–500 bp). Their resolving power is extremely high, and fragments of DNA that differ in size by as little as 1 bp in length or by as little as 0.1% of their mass can be separated from one another. Although they can be run very rapidly and can accommodate comparatively large quantities of DNA, polyacrylamide gels have the disadvantage of being more difficult to prepare and handle than agarose gels. Polyacrylamide gels are run in a vertical configuration in a constant electric field.

Agarose gels have a lower resolving power than polyacrylamide gels, but they have a greater range of separation. DNAs from 50 bp to several megabases in length can be separated on agarose gels of various concentrations and configurations. Small DNA fragments (50–20,000 bp) are best resolved in agarose gels run in a horizontal configuration in an electric field of constant strength and direction. Under these conditions, the velocity of the DNA fragments decreases as their length increases and is proportional to electric field strength (McDonell et al. 1977; Fangman 1978; Calladine et al. 1991). However, this simple relationship breaks down once the size of DNA fragments exceeds a maximum value, which is defined chiefly by the composition of the gel and the strength of the electric field (Hervet and Bean 1987). This limit of resolution is reached when the radius of gyration of the linear DNA duplex exceeds the pore size of the gel. The DNA can then no longer be sieved by the gel according to its size but must instead migrate "end-on" through the matrix as if through a sinuous tube. This mode of migration is known as "reptation." Several mathematical descriptions of reptation have been published previously (please see Lerman and Frisch 1982; Lumpkin and Zimm 1982; Stellwagen 1983; Edmondson and Gray 1984; Slater and Noolandi 1985, 1986; Lalande et al. 1987).

The greater the pore size of the gel, the larger the DNA that can be sieved. Thus, agarose gels cast with low concentrations of agarose (0.1–0.2% w/v) are capable of resolving extremely large DNA molecules (Fangman 1978; Serwer 1980). However, such gels are extremely fragile and must be run for several days. Even then, they are incapable of resolving linear DNA molecules larger than 750 kb in length. The importance of this limitation becomes apparent with the realization that a single genetic locus (e.g., the human dystrophin locus) may occupy several thousand kilobases (several megabases) of DNA and that DNA molecules in the individual chromosomes of lower eukaryotes may be 7000 kb or more in length.

A solution to this problem was found in 1984, when Schwartz and Cantor reported the development of pulsed-field gel electrophoresis (PFGE). In this method, alternating orthogonal electric fields are applied to a gel. Large DNA molecules become trapped in their reptation tubes every time the direction of the electric field is altered and can make no further progress through the gel until they have reoriented themselves along the new axis of the electric field. The larger the DNA molecule, the longer the time required for this realignment. Molecules of DNA whose

reorientation times are less than the period of the electric pulse will therefore be fractionated according to size. The limit of resolution of PFGE depends on several factors, including:

- the degree of uniformity of the two electric fields
- the absolute lengths of the electric pulses
- the angles between the two electric fields
- the relative strength of the electric field

The original PFGE method described by Schwartz and Cantor (1984) was capable of resolving DNAs up to 2000 kb in length. However, as a consequence of improvements to the technique, resolution of DNA molecules larger than 6000 kb can now be achieved. These developments mean that PFGE can be used to determine the size of bacterial genomes and the numbers and sizes of chromosomes of simpler eukaryotes (e.g., *Neurospora crassa, Saccharomyces cerevisiae,* and *Schizosaccharomyces pombe*). For all organisms, from bacteria to humans, PFGE is used to study genome organization and to clone and analyze large fragments.

EARLY ANALYSIS OF DNA USING ELECTROPHORESIS

The idea of using electrophoresis through a supporting matrix to analyze DNA came from Vin Thorne, a biochemist/virologist who in the mid-1960s was working at the Institute of Virology in Glasgow. Thorne was interested in finding better ways to characterize the multiple forms of DNA that could be extracted from purified particles of polyomavirus. He reasoned that a combination of frictional and electrical forces would allow separation of DNA molecules that differed in shape or size. Using electrophoresis through agar gels, he was able to separate superhelical, nicked, and linear forms of polyomavirus DNA that had been radiolabeled with [^{3}H]thymidine (Thorne 1966, 1967). In those days, viral and mitochondrial DNAs were the only intact genomes that could be prepared in pure form. Thorne's work therefore attracted little general interest until the early 1970s when restriction enzymes opened the possibility of analyzing larger DNAs, and a way was found to detect small quantities of nonradioactive DNA in gels.

The notion of using ethidium bromide to stain unlabeled DNA in gels seems to have occurred independently to two groups. The procedure used by Aaij and Borst (1972) involved immersing the gel in concentrated dye solution and a lengthy destaining process to reduce the background fluorescence. At Cold Spring Harbor Laboratory, a group of investigators had found that *Haemophilus parainfluenzae* contained two restriction activities and were attempting to separate the enzymes by ion-exchange chromatography. Searching for ways to assay column fractions rapidly, they decided to stain agarose gels containing fragments of SV40 DNA with low concentrations of ethidium bromide. They soon realized that the dye could be incorporated into the gel and running buffer without significantly affecting the migration of linear DNA fragments through the gel. The technique described in their paper (Sharp et al. 1973) is still widely used in an essentially unaltered form today.

Between 1972 and 1975, there was a vast increase in the use of agarose gels as investigators mapped cleavage sites on their favorite DNAs with the rapidly expanding suite of restriction enzymes. In those days, gels were cast in sawn-off glass pipettes and were run vertically in electrophoresis tanks attached to homemade Heathkit power packs. Each DNA sample was analyzed on a separate little cylindrical gel. The first modern electrophoresis apparatus was developed by Walter Schaffner, who was then a graduate student at Zurich. Realizing that the electrical resistance of an agarose gel is essentially the same as that of the surrounding buffer, Schaffner constructed horizontal tanks to hold submerged gels that could accommodate more than a dozen samples. Schaffner distributed the plans for these machines to anyone who asked for them. Once people got over their incredulity that his machines actually worked, cylindrical gels cast in little glass tubes rapidly disappeared, and the newer "submarine" gels took hold.

Protocol 1

Agarose Gel Electrophoresis

AGAROSE IS A LINEAR POLYMER COMPOSED OF ALTERNATING RESIDUES of D- and L-galactose joined by α-(1→3) and β-(1→4) glycosidic linkages. The L-galactose residue has an anhydro bridge between the three and six positions (please see Figure 5-1). Chains of agarose form helical fibers that aggregate into supercoiled structures with a radius of 20–30 nm. Gelation of agarose results in a three-dimensional mesh of channels whose diameters range from 50 nm to >200 nm (Norton et al. 1986; for review, please see Kirkpatrick 1990).

Commercially prepared agarose polymers are believed to contain ~800 galactose residues per chain. However, agarose is not homogeneous: The average length of the polysaccharide chains varies from batch to batch and from manufacturer to manufacturer. In addition, lower grades of agarose may be contaminated with other polysaccharides, as well as salts and proteins. This variability can affect the gelling/melting temperature of agarose solutions, the sieving of DNA, and the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions. These potential problems can be minimized by using special grades of agarose that are screened for the presence of inhibitors and nucleases and for minimal background fluorescence after staining with ethidium bromide.

THE RATE OF MIGRATION OF DNA THROUGH AGAROSE GELS

The following factors determine the rate of migration of DNA through agarose gels:

- ***The molecular size of the DNA.*** Molecules of double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs (Helling et al. 1974). Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules.

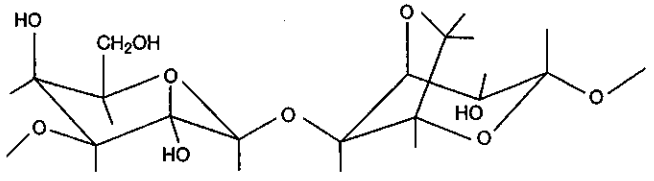

FIGURE 5.1 Chemical Structure of Agarose

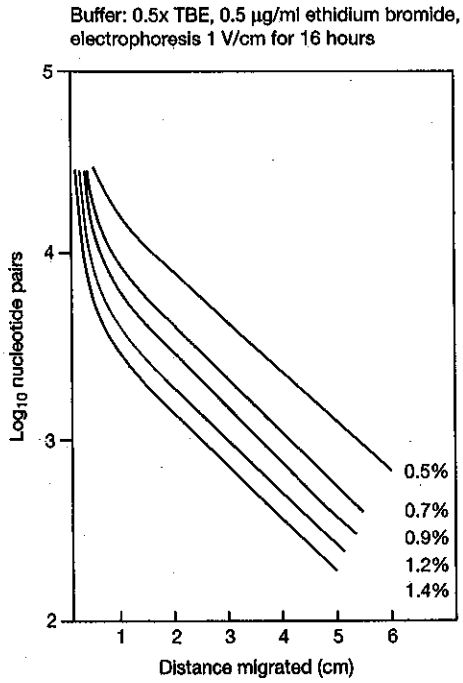

FIGURE 5-2 The Relationship between the Size of DNA and Its Electrophoretic Mobility

- ***The concentration of agarose.*** A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose (please see Figure 5-2). There is a linear relationship between the logarithm of the electrophoretic mobility of the DNA (μ) and the gel concentration (ι) that is described by the equation:

  $$\log \mu = \log \mu_o - K_r \iota$$

  where μ_o is the free electrophoretic mobility of DNA and K_r is the retardation coefficient, a constant related to the properties of the gel and the size and shape of the migrating molecules.

- ***The conformation of the DNA.*** Superhelical circular (form I), nicked circular (form II), and linear (form III) DNAs migrate through agarose gels at different rates (Thorne 1966, 1967). The relative mobilities of the three forms depend primarily on the concentration and type of agarose used to make the gel, but they are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of superhelical twists in the form I DNA (Johnson and Grossman 1977). Under some conditions, form I DNA migrates faster than form III DNA; under other conditions, the order is reversed. In most cases, the best way to distinguish between the different conformational forms of DNA is simply to include in the gel a sample of untreated circular DNA and a sample of the same DNA that has been linearized by digestion with a restriction enzyme that cleaves the DNA in only one place.

- ***The presence of ethidium bromide in the gel and electrophoresis buffer.*** Intercalation of ethidium bromide causes a decrease in the negative charge of the double-stranded DNA and an increase in both its stiffness and length. The rate of migration of the linear DNA-dye complex through gels is consequently retarded by a factor of ~15% (Sharp et al. 1973).

- ***The applied voltage.*** At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the strength of the electric field is raised, the mobility of high-molecular-weight fragments increases differentially. Thus, the effective range

TABLE 5-1 Properties of Different Types of Agaroses

Type of Agarose	Gelling Temperature (°C)	Melting Temperature (°C)	Commercial Names
Standard agaroses			
low EEO	35–38	90–95	SeaKem LE (BioWhittaker)
isolated from *Gelidium* spp.			Agarose-LE (USB)
			Low EEO Agarose (Stratagene)
			Molecular Biology Certified Grade (Bio-Rad)
Standard agaroses			
low EEO	40–42	85–90	SeaKem HGT (BioWhittaker)
isolated from *Gracilaria* spp.			Agarose-HGT (USB)
High-gel-strength agaroses	34–43	85–95	FastLane (BioWhittaker)
			SeaKem Gold (BioWhittaker)
			Chromosomal Grade Agarose (Bio-Rad)
Low melting/gelling temperature (modified) agaroses			
low melting	25–35	63–65	SeaPlaque (BioWhittaker)
	35	65	NuSieve GTG (BioWhittaker)
ultra-low melting	8–15	40–45	SeaPrep (BioWhittaker)
Low-viscosity, low melting/gelling temperature agaroses			
	25–30	70	InCert (BioWhittaker)
	38	85	NuSieve 3:1 (BioWhittaker)
	30	75	Agarose HS (BioWhittaker)

of separation in agarose gels decreases as the voltage is increased. To obtain maximum resolution of DNA fragments >2 kb in size, agarose gels should be run at no more than 5–8 V/cm.

- ***The type of agarose.*** The two major classes of agarose are standard agaroses and low-melting-temperature agaroses (Kirkpatrick 1990). A third and growing class consists of intermediate melting/gelling temperature agaroses, exhibiting properties of each of the two major classes. Within each class are various types of agaroses that are used for specialized applications, please

TABLE 5-2 Range of Separation of DNA Fragments through Different Types of Agaroses

	Size Range of DNA Fragments Resolved by Various Types of Agaroses			
Agarose (%)	Standard	High Gel Strength	Low Gelling/Melting Temperature	Low Gelling/Melting Temperature Low Viscosity
0.3				
0.5	700 bp to 25 kb			
0.8	500 bp to 15 kb	800 bp to 10 kb	800 bp to 10 kb	
1.0	250 bp to 12 kb	400 bp to 8 kb	400 bp to 8 kb	
1.2	150 bp to 6 kb	300 bp to 7 kb	300 bp to 7 kb	
1.5	80 bp to 4 kb	200 bp to 4 kb	200 bp to 4 kb	
2.0		100 bp to 3 kb	100 bp to 3 kb	
3.0			500 bp to 1 kb	500 bp to 1 kb
4.0				100 bp to 500 bp
6.0				10 bp to 100 bp

see Tables 5-1 and 5-2 and the accompanying panel, **CLASSES OF AGAROSE AND THEIR PROPERTIES.**

- ***The electrophoresis buffer.*** The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions (e.g., if water is substituted for electrophoresis buffer in the gel or in the reservoirs), electrical conductivity is minimal

CLASSES OF AGAROSE AND THEIR PROPERTIES

- ***Standard (high-melting-temperature) agaroses*** are manufactured from two species of seaweed: *Gelidium* and *Gracilaria*. These agaroses differ in their gelling and melting temperatures, but, for practical purposes, agaroses from either source can be used to analyze and isolate fragments of DNA ranging in size from 1 kb to 25 kb. Several commercial grades of agaroses have been tested that (1) display minimal background fluorescence after staining with ethidium bromide, (2) are free of DNase and RNase, (3) display minimal inhibition of restriction endonucleases and ligase, and (4) generate modest amounts of electroendo-osmotic flow (EEO; please see below).

  Newer types of standard agarose combine high gel strength with low EEO, allowing gels to be cast with agarose concentrations as low as 0.3%. These gels can be used in conventional electrophoresis to separate high-molecular-weight DNA (up to 60 kb). At any concentration of these new agaroses, the speed of migration of the DNA is increased by 10–20% over that achieved using the former standard agaroses, depending on buffer type and concentration. This increase can lead to significant savings of time in PFGE of megabase-sized DNA.

- ***Low melting/gelling temperature agaroses*** have been modified by hydroxyethylation and therefore melt at temperatures lower than those of standard agaroses. The degree of substitution determines the exact melting and gelling temperature. Low melting/gelling temperature agaroses are used chiefly for rapid recovery of DNA, as most agaroses of this type melt at temperatures (~65°C) that are significantly lower than the melting temperature of duplex DNA. This feature allows for simple purification, enzymatic processing (restriction endonuclease digestion/ligation) of DNA; and allows bacterial transformation with nucleic acids directly in the remelted gel. As is the case with standard agaroses, manufacturers provide grades of low-melting-temperature agaroses that have been tested to display minimal background fluorescence after staining with ethidium bromide, to be free of DNase and RNase activity, and to display minimal inhibition of restriction endonucleases and ligase. Low-melting-temperature agaroses not only melt, but also gel at low temperatures. This property allows them to be held as liquids in the 30–35°C range, so that cells can be embedded without damage. This treatment is useful in preparing and embedding chromosomal DNA in agarose blocks before analysis by PFGE (please see Protocols 13 and 14).

  Chemically modified agarose has significantly more sieving capacity than an equivalent concentration of standard agarose (please see Tables 5-1 and 5-2). This finding has been exploited to make agaroses that approach polyacrylamide in their resolving power and are therefore useful for separation of polymerase chain reaction (PCR) products, small DNA fragments, and small RNAs <1 kb. It is now possible to resolve DNA down to 4 bp and to separate DNAs in the 200–800-bp range that differ in size by 2% (please see Table 5-2).

  Because of the variation in products from manufacturer to manufacturer, it is advisable to read the supplier's catalog to obtain more precise information about specific brands of agarose.

- ***Electroendo-osmosis.*** In agarose gels, the speed at which nucleic acids migrate toward the positive electrode is affected by a electroendo-osmosis. This process is due to ionized acidic groups (usually sulfate) attached to the polysaccharide matrix of the agarose gel. The acidic groups induce positively charged counterions in the buffer that migrate through the gel toward the negative electrode, causing a bulk flow of liquid that migrates in a direction opposite to that of the DNA.

  The higher the density of negative charge on the agarose, the greater the EEO flow and the poorer the separation of nucleic acid fragments. Retardation of small DNA fragments (<10 kb) is minor, but larger DNA molecules can be significantly retarded, especially in PFGE. To avoid problems, it is best to purchase agarose from reputable merchants and to use types of agarose that display low levels of EEO. Agaroses that are sold as "zero" EEO are undesirable for two reasons: They have been chemically modified by adding positively charged groups, which neutralize the sulfated polysaccharides in the gel but may inhibit subsequent enzyme reactions, and they have been adulterated by adding locust bean gum, which retards expulsion of water from the gel (Kirkpatrick 1990).

and DNA migrates slowly, if at all. In buffer of high ionic strength (e.g., if 10x electrophoresis buffer is mistakenly used), electrical conductance is very efficient and significant amounts of heat are generated, even when moderate voltages are applied. In the worst case, the gel melts and the DNA denatures. For details of commonly used electrophoresis buffers, please see Table 5-3.

ELECTROPHORESIS BUFFERS

Several different buffers are available for electrophoresis of native, double-stranded DNA. These contain Tris-acetate and EDTA (pH 8.0; TAE) (also called E buffer), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of ~50 mM (pH 7.5–7.8). Electrophoresis buffers are usually made up as concentrated solutions and stored at room temperature (please see Table 5-3).

All of these buffers work well, and the choice among them is largely a matter of personal preference. TAE has the lowest buffering capacity of the three and will become exhausted if electrophoresis is carried out for prolonged periods of time. When this happens, the anodic portion of the gel becomes acidic and the bromophenol blue migrating through the gel toward the anode changes in color from bluish-purple to yellow. This change begins at pH 4.6 and is complete at pH 3.0. Exhaustion of TAE can be avoided by periodic replacement of the buffer during electrophoresis or by recirculation of the buffer between the two reservoirs. Both TBE and TPE are slightly more expensive than TAE, but they have significantly higher buffering capacity. Double-stranded linear DNA fragments migrate ~10% faster through TAE than through TBE or TPE; the resolving power of TAE is slightly better than TBE or TPE for high-molecular-weight DNAs and worse for low-molecular-weight DNAs. This difference probably explains the observation that electrophoresis in TAE yields better resolution of DNA fragments in highly complex mixtures such as mammalian DNA. For this reason, Southern blots used to analyze complex genomes are generally derived from gels prepared in and run with TAE as the electrophoresis buffer. The resolution of supercoiled DNAs is better in TAE than in TBE.

TABLE 5-3 Electrophoresis Buffers

BUFFER	WORKING SOLUTION	STOCK SOLUTION/LITER
TAE	1X	50X
	40 mM Tris-acetate	242 g of Tris base
	1 mM EDTA	57.1 ml of glacial acetic acid
		100 ml of 0.5 M EDTA (pH 8.0)
TPE	1X	10X
	90 mM Tris-phosphate	108 g of Tris base
	2 mM EDTA	15.5 ml of phosphoric acid (85%, 1.679 g/ml)
		40 ml of 0.5 M EDTA (pH 8.0)
TBE[a]	0.5X	5X
	45 mM Tris-borate	54 g of Tris base
	1 mM EDTA	27.5 g of boric acid
		20 ml of 0.5 M EDTA (pH 8.0)

[a]TBE is usually made and stored as a 5x or 10x stock solution. The pH of the concentrated stock buffer should be ~8.3. Dilute the concentrated stock buffer just before use and make the gel solution and the electrophoresis buffer from the same concentrated stock solution. Some investigators prefer to use more concentrated stock solutions of TBE (10x as opposed to 5x). However, 5x stock solution is more stable because the solutes do not precipitate during storage. Passing the concentrated buffer stocks through a 0.45-μm filter can prevent or delay formation of precipitates.

TABLE 5-4 6x Gel-loading Buffers

BUFFER TYPE	6X BUFFER	STORAGE TEMPERATURE
I	0.25% bromophenol blue 0.25% xylene cyanol FF 40% (w/v) sucrose in H_2O	4°C
II	0.25% bromophenol blue 0.25% xylene cyanol FF 15% Ficoll (Type 400; Pharmacia) in H_2O	room temperature
III	0.25% bromophenol blue 0.25% xylene cyanol FF 30% glycerol in H_2O	4°C
IV	0.25% bromophenol blue 40% (w/v) sucrose in H_2O	4°C

GEL-LOADING BUFFERS

Gel-loading buffers are mixed with the samples before loading into the slots of the gel. These buffers serve three purposes: They increase the density of the sample, ensuring that the DNA sinks evenly into the well; they add color to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol blue migrates through agarose gels ~2.2-fold faster than xylene cyanol FF, independent of the agarose concentration. Bromophenol blue migrates through agarose gels run in 0.5x TBE at approximately the same rate as linear double-stranded DNA 300 bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear double-stranded DNA 4 kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5–1.4%. Which type of loading dye to use is a matter of personal preference; various recipes are presented in Table 5-4.

MATERIALS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

Agarose solutions (Tables 5-1 and 5-2)

Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. For rapid analysis of DNA samples, the use of a minigel is recommended (please see the panel on **ELECTROPHORESIS THROUGH MINIGELS** below Step 13).

Electrophoresis buffer (usually 1x TAE or 0.5x TBE)

Please see Table 5-3 for recipes.

Ethidium bromide <!> or SYBR Gold staining solution <!>

For a discussion of staining DNA in agarose gels, please see Protocol 2.

6x Gel-loading buffer

Please see Table 5-4 for recipes.

Nucleic Acids and Oligonucleotides

DNA samples

DNA size standards

Samples of DNAs of known size are typically generated by restriction enzyme digestion of a plasmid or bacteriophage DNA of known sequence. Alternatively, they are produced by ligating a monomer DNA fragment of known size into a ladder of polymeric forms. Size standards for both agarose and polyacrylamide gel electrophoresis may be purchased from commercial sources or they can be prepared easily in the laboratory. It is a good idea to have two size ranges of standards, including a high-molecular-weight range from 1 kb to >20 kb and a low-molecular-weight range from 100 bp to 1000 bp. A stock solution of size standards can be prepared by dilution with a gel-loading buffer and then used as needed in individual electrophoresis experiments.

Special Equipment

Equipment for agarose gel electrophoresis

Clean, dry horizontal electrophoresis apparatus with chamber and comb, or clean dry glass plates with appropriate comb.

Gel-sealing tape

Common types of lab tape, such as Time tape or VWR lab tape, are appropriate for sealing the ends of the agarose gel during pouring.

Microwave oven or Boiling water bath

Power supply device capable of up to 500 V and 200 mA.

Water bath preset to 55°C

METHOD

1. Seal the edges of a clean, dry glass plate (or the open ends of the plastic tray supplied with the electrophoresis apparatus) with tape to form a mold (Figure 5-3). Set the mold on a horizontal section of the bench.

2. Prepare sufficient electrophoresis buffer (usually 1x TAE or 0.5x TBE) to fill the electrophoresis tank and to cast the gel.

   It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel. Small differences in ionic strength or pH create fronts in the gel that can greatly affect the mobility of DNA fragments. When measuring the sizes of unknown DNAs, ensure that all samples are applied to the gel in the same buffer. The high concentrations of salt in certain restriction enzyme buffers (e.g., *Bam*HI and *Eco*RI) retard the migration of DNA and distort the electrophoresis of DNA in the adjacent wells.

3. Prepare a solution of agarose in electrophoresis buffer at a concentration appropriate for separating the particular size fragments expected in the DNA sample(s): Add the correct amount of powdered agarose (please see Table 5-5) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle.

   The buffer should occupy less than 50% of the volume of the flask or bottle.

   The concentrations of agarose required to separate DNAs in different size ranges is given in Table 5-2. DNAs differing in size by only a few base pairs can be separated when certain high-resolution agaroses (e.g., MetaPhor agarose, BioWhittaker) are used to cast the gel. Alternatively, modified polysaccharides (commerically available) can be added to regular agarose to enhance separation. This substance, used at a concentration of 0.5–2.0% (w/v), together with agarose, increases resolution, renders the cast gel more clear, and increases the strength of the gel.

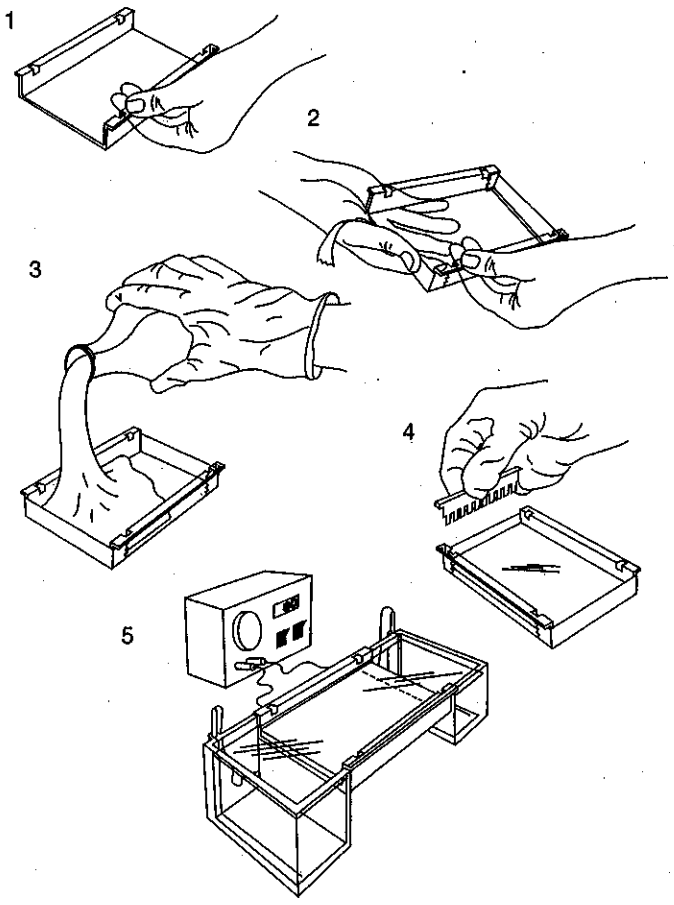

FIGURE 5-3 Pouring a Horizontal Agarose Gel

4. Loosely plug the neck of the Erlenmeyer flask with Kimwipes. If using a glass bottle, make certain the cap is loose. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves.

   ▲ **WARNING** The agarose solution can become superheated and may boil violently if it is heated for too long in the microwave oven.

   Heat the slurry for the minimum time required to allow all of the grains of agarose to dissolve. Undissolved agarose appears as small "lenses" or translucent chips floating in the solution. Wear an oven mitt and carefully swirl the bottle or flask from time to time to make sure that any unmelted grains of agarose sticking to the walls enter the solution. Longer heating times are required to dissolve higher concentrations of agarose completely. Check that the volume of the solution has not been decreased by evaporation during boiling; replenish with H_2O if necessary.

5. Use insulated gloves or tongs to transfer the flask/bottle into a water bath at 55°C. When the molten gel has cooled, add ethidium bromide to a final concentration of 0.5 µg/ml. Mix the gel solution thoroughly by gentle swirling.

   ▲ **IMPORTANT** SYBR Gold should not be added to the molten gel solution. Please see Protocol 2 for further discussion.

   When preparing gels in plastic (lucite) trays, it is important to cool the melted agarose solution to <60°C before casting the gel. Hotter solutions warp and craze the trays. At one time, solutions con-

TABLE 5-5 Range of Separation in Gels Containing Different Amounts of Standard Low-EEO Agarose

Agarose Concentration in Gel (% [W/V])	Range of Separation of Linear DNA Molecules (kb)
0.3	5–60
0.6	1–20
0.7	0.8–10
0.9	0.5–7
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2

taining high concentrations of agarose (2% or above) were stored at 70°C to prevent premature gelling. However, this treatment has become unnecessary because of improvements in the methods used to purify and prepare standard agaroses.

6. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel. Position the comb 0.5–1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.

   Most apparatuses have side walls or outside "legs" that allow appropriate placement of the comb. If this is not the case, and if the comb is too close to the glass plate, the base of the well may tear when the comb is withdrawn, causing samples to leak between the gel and the glass plate. This problem is more common when low concentrations of agarose (<0.6%) or low-gelling-temperature agarose are used.

7. Pour the warm agarose solution into the mold.

   The gel should be between 3 mm and 5 mm thick. Check that no air bubbles are under or between the teeth of the comb. Air bubbles present in the molten gel can be removed easily by poking them with the corner of a Kimwipe.

   When preparing gels that contain low concentrations of agarose (<0.5%), first pour a supporting gel (1% agarose) without wells. Allow this gel to harden at room temperature on the glass plate or plastic tray, and then pour the lower-percentage gel directly on top of the supporting gel. Stacking the gels in this way reduces the chance that the lower-percentage gel will fracture during subsequent manipulations (e.g., photography and processing for Southern hybridization). Make sure that both gels are made from the same batch of buffer and contain the same concentration of ethidium bromide. Gels cast with low-melting-temperature agarose and gels that contain less than 0.5% agarose can also be chilled to 4°C and run in a cold room to reduce the chance of fracture.

8. Allow the gel to set completely (30–45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer and carefully remove the tape. Mount the gel in the electrophoresis tank.

9. Add just enough electrophoresis buffer to cover the gel to a depth of ~1 mm.

   It is not necessary to prerun an agarose gel before the samples are loaded.

10. Mix the samples of DNA with 0.20 volume of the desired 6× gel-loading buffer (please see Table 5-4).

    The maximum amount of DNA that can be applied to a slot depends on the number of fragments in the sample and their sizes. The minimum amount of DNA that can be detected by photography of ethidium-bromide-stained gels is ~2 ng in a 0.5-cm-wide band (the usual width of a slot). More sensitive dyes such as SYBR Gold can detect as little as 20 pg of DNA in a band. If there is more than 500 ng of DNA in a band of 0.5 cm, the slot will be overloaded, resulting in trailing, smiling, and smearing — problems that become more severe as the size of the DNA increases. When simple populations of DNA molecules (e.g., bacteriophage λ or plasmid DNAs) are to be analyzed, 100–500 ng of DNA should be loaded per 0.5-cm slot. When the sample consists of a very large number of DNA fragments of different sizes (e.g., restriction digests of mammalian DNA), however, it is possible to load 20–30 μg of DNA per slot without significant loss of resolution.

The maximum volume of solution that can be loaded is determined by the dimensions of the slot. (A typical slot [0.5 x 0.5 x 0.15 cm] will hold about 40 µl). Do not overfill a slot with a DNA sample solution. To reduce the possibility of contaminating neighboring samples, it is best to make the gel a little thicker or to concentrate the DNA by ethanol precipitation rather than to fill the slot completely.

11. Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipettor, or a drawn-out Pasteur pipette or glass capillary tube. Load size standards into slots on both the right and left sides of the gel.

For many purposes, it is not necessary to use a fresh pipette tip for every sample as long as the tip is thoroughly washed with buffer from the anodic chamber between samples. However, if the gel is to be analyzed by Southern hybridization or if bands of DNA are to be recovered from the gel, it is sensible to use a separate pipette tip for every sample.

12. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1–5 V/cm (measured as the distance between the positive and negative electrodes). If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis), and within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and xylene cyanol FF have migrated an appropriate distance through the gel.

The presence of ethidium bromide allows the gel to be examined by UV illumination at any stage during electrophoresis. The gel tray may be removed and placed directly on a transilluminator. Alternatively, the gel may be examined using a hand-held source of UV light. In either case, turn off the power supply before examining the gel!

During electrophoresis, the ethidium bromide migrates toward the cathode (in the direction opposite to that of the DNA). Electrophoresis for protracted periods of time can result in the loss of significant amounts of ethidium bromide from the gel, making detection of small fragments difficult. In this case, restain the gel by soaking it for 30–45 minutes in a solution of ethidium bromide (0.5 µg/ml) as described in Protocol 2.

13. When the DNA samples or dyes have migrated a sufficient distance through the gel, turn off the electric current and remove the leads and lid from the gel tank. If ethidium bromide is present in the gel and electrophoresis buffer, examine the gel by UV light and photograph the gel as described in Protocol 2 and as shown in Figure 5-4. Otherwise, stain the gel by immersing it in electrophoresis buffer or H_2O containing ethidium bromide (0.5 µg/ml) for 30–45 minutes at room temperature or by soaking in a 1:10,000-fold dilution of SYBR Gold stock solution in electrophoresis buffer.

For further details on staining and photography of DNA in gels, please see Protocol 2.

ELECTROPHORESIS THROUGH MINIGELS

During the last several years, methods have been developed for analyzing small quantities of DNA very rapidly using agarose minigels. Several types of miniature electrophoresis tanks are manufactured commercially, typically as smaller versions of the companies' larger electrophoresis models. Each gel slot holds 3–12 µl of sample, depending on the thickness of the gel and the width of the teeth of the comb. Usually, 10–100 ng of DNA in the gel-loading buffer of choice is applied to a slot. The gel is then run for 30–60 minutes at high voltage (5–20 V/cm) until the bromphenol blue and xylene cyanol FF have migrated the appropriate distance. The gel is then photographed as described in Protocol 2.

Minigels are particularly useful when a rapid answer is required before the next step in a cloning protocol can be undertaken. Because the wells are smaller and the gels thinner, less DNA than normal is required for visualization. In addition, because the gels can be prepared in advance and run rapidly, and because they require smaller amounts of reagents, there are considerable savings in both time and money. Many investigators prepare one gel at the beginning of the week, and use it over and over again through the course of an experiment. Thus, a particular set of samples may be loaded, run out onto the gel, and visualized. The gel then may be "erased" by running the samples off the gel into the buffer. Note that minigels are best suited for the analysis of small DNA fragments (<3 kb). Larger fragments resolve poorly because of the high voltages that are generally used and the comparatively short length of the gel.

Protocol 2

Detection of DNA in Agarose Gels

NUCLEIC ACIDS THAT HAVE BEEN SUBJECTED TO ELECTROPHORESIS through agarose gels may be detected by staining and visualized by illumination with 300-nm UV light. Methods for staining and visualization of DNA using either ethidium bromide or SYBR Gold are described here; for further details on detection of nucleic acids, please see the discussion on the Quantitation of Nucleic Acids in Appendix 8.

STAINING DNA IN GELS USING ETHIDIUM BROMIDE

The most convenient and commonly used method to visualize DNA in agarose gels is staining with the fluorescent dye ethidium bromide (Sharp et al. 1973), which contains a tricyclic planar group that intercalates between the stacked bases of DNA. Ethidium bromide binds to DNA with little or no sequence preference. At saturation in solutions of high ionic strength, approximately one ethidium molecule is intercalated per 2.5 bp (Waring 1965). After insertion into the helix, the dye lies perpendicular to the helical axis and makes van der Waals contacts with the base pairs above and below. The fixed position of the planar group and its close proximity to the bases cause dye bound to DNA to display an increased fluorescent yield compared to that of dye in free solution. UV radiation at 254 nm is absorbed by the DNA and transmitted to the dye; radiation at 302 nm and 366 nm is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum (LePecq and Paoletti 1967). Because the fluorescent yield of ethidium bromide–DNA complexes is ~20–30-fold greater than that of unbound dye, bands containing as little as ~10 ng of DNA can be detected in the presence of free ethidium bromide (0.5 μg/ml) in the gel.

> Ethidium bromide was synthesized in the 1950s in an effort to develop phenanthridine compounds as effective trypanocidal agents. Ethidium emerged from the screening program with flying colors. It was 10–50-fold more effective against trypanosomes than the parent compound, was no more toxic to mice, and, unlike earlier phenanthridines, did not induce photosensitization in cattle (Watkins and Woolfe 1952). Ethidium bromide is still used for the treatment and prophylaxis of trypanomiasis in cattle in tropical and subtropical countries.

Ethidium bromide can be used to detect both single- and double-stranded nucleic acids (both DNA and RNA). However, the affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is comparatively poor. In fact, most fluorescence associated with staining single-stranded DNA or RNA is attributable to binding of the dye to short

intrastrand duplexes in the molecules. For additional details on ethidium bromide, please see the information panel on **ETHIDIUM BROMIDE** in Chapter 1.

Ethidium bromide is prepared as a stock solution of 10 mg/ml in H_2O, which is stored at room temperature in dark bottles or bottles wrapped in aluminum foil. The dye is usually incorporated into agarose gels and electrophoresis buffers at a concentration of 0.5 μg/ml. Note that polyacrylamide gels cannot be cast with ethidium bromide because it inhibits polymerization of the acrylamide. Acrylamide gels are therefore stained with the ethidium solution after the gel has been run (please see Protocol 10).

Although the electrophoretic mobility of linear double-stranded DNA is reduced by ~15% in the presence of the dye, the ability to examine the agarose gels directly under UV illumination during or at the end of the run is a great advantage. However, sharper DNA bands are obtained when electrophoresis is carried out in the absence of ethidium bromide. Thus, when an accurate size of a particular fragment of DNA is to be established (e.g., when a restriction endonuclease map is being determined for a fragment of DNA), the agarose gel should be run in the absence of ethidium bromide and stained after electrophoresis is complete. Staining is accomplished by immersing the gel in electrophoresis buffer or H_2O containing ethidium bromide (0.5 μg/ml) for 30–45 minutes at room temperature. Destaining is not usually required. However, detection of very small amounts (<10 ng) of DNA is made easier if the background fluorescence caused by unbound ethidium bromide is reduced by soaking the stained gel in H_2O or 1 mM $MgSO_4$ for 20 minutes at room temperature.

STAINING DNA IN GELS USING SYBR GOLD

SYBR Gold is the trade name of a new ultrasensitive dye with high affinity for DNA and a large fluorescence enhancement upon binding to nucleic acid. The quantum yield of the SYBR Gold–DNA complex is greater than that of the equivalent ethidium bromide–DNA complex and the fluorescence enhancement is >1000 times greater. As a result, <20 pg of double-stranded DNA can be detected in an agarose gel (up to 25 times less than the amount visible after ethidium bromide staining). In addition, staining of agarose or polyacrylamide gels with this dye can reveal as little as 100 pg of single-stranded DNA in a band or 300 pg of RNA. SYBR Gold shows maximum excitation at 495 nm and has a secondary excitation peak at 300 nm. Fluorescent emission occurs at 537 nm. For additional details on SYBR Gold, please see the panel below.

SYBR GOLD

SYBR Gold is a proprietary fluorescent unsymmetrical cyanine dye, sold by Molecular Probes, that is used to stain single- and double-stranded nucleic acids in gels. Although far more expensive, SYBR Gold has several advantages over phenanthridine dyes such as ethidium bromide. It is more sensitive and can be used to stain both DNA and RNA in conventional neutral polyacrylamide and agarose gels and in gels containing denaturants, such as urea, glyoxal, and formaldehyde. When excited by standard transillumination at 300 nm, SYBR Gold gives rise to bright gold fluorescent signals that can be captured on conventional black and white Polaroid film or on charged couple device (CCD)-based image detection systems. Gels are stained with SYBR Gold after electrophoresis is complete. The level of background fluorescence is so low that no destaining is required. The stained nucleic acid can be transferred directly to membranes for northern or Southern hybridization. SYBR Gold may be removed from nucleic acids recovered from gels by ethanol precipitation.

SYBR Gold is supplied as a 10,000x concentrate in anhydrous dimethylsulfoxide (DMSO). The high cost of the dye precludes its use for routine staining of gels. However, the dye may be cost-effective as an alternative to using radiolabeled DNAs in techniques such as single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE).

SYBR Gold is used to stain DNA by soaking the gel, after separation of the DNA fragments, in a 1:10,000-fold dilution of the stock dye solution. SYBR Gold should not be added to the molten agarose or to the gel before electrophoresis, because its presence in the hardened gel will cause severe distortions in the electrophoretic properties of the DNA and RNA.

The greatest sensitivity is obtained when the gel is illuminated with UV light at 300 nm. Photography is carried out as described below with green or yellow filters. The dye is sensitive to fluorescent light, and working solutions containing SYBR Gold (1:10,000 dilution of the stock solution supplied by Molecular Probes) should be freshly made daily in electrophoresis buffer and stored at room temperature.

PHOTOGRAPHY OF DNA IN GELS

Photographs of ethidium-bromide-stained gels may be made using transmitted or incident UV light (please see Figure 5-4). Most commercially available devices (transilluminators) emit UV light at 302 nm. The fluorescent yield of ethidium bromide–DNA complexes is considerably greater at this wavelength than at 366 nm and slightly less than at short-wavelength (254 nm) light. However, the amount of nicking of the DNA is much less at 302 nm than at 254 nm (Brunk and Simpson 1977).

Today, images of ethidium-bromide-stained gels may be captured by integrated systems containing light sources, fixed-focus digital cameras, and thermal printers. The CCD cameras of these systems use a wide-angle zoom lens (f = 75 mm) that allows the detection of very small amounts of ethidium-bromide-stained DNA (0.01–0.5 ng is claimed). In the more advanced systems, gel images are directly transmitted to a computer and visualized in real time. The image can be manipulated on screen with respect to field, focus, and cumulative exposure time prior to printing. Individual images can be printed, saved, and stored electronically in several file formats and further manipulated with image analysis software programs. The average file size for a stained agarose gel image is ~0.3 Mb; thus, extensive archiving requires large-capacity storage systems. Although individual printouts cost only a few pennies compared to ~1 dollar for a Polaroid photograph (please see below), the hardware for a minimum integrated system can cost several thousand dollars and considerably more for a setup with a large assortment of accessories. Vendors that sell gel documentation systems include Alpha Innotech (San Leandro, California), Fotodyne (Hartland, Wisconsin), and Stratagene (La Jolla, California).

Although the results obtained with these documentation systems are entirely satisfactory for immediate analysis, the printed images fade during storage and are devoid of esthetic appeal. More pleasing and durable results are obtained from highly sensitive Polaroid film Type 57 or 667 (ASA 3000). With an efficient UV light source (>2500 mW/cm^2), a Wratten 22A (red/orange) filter, and a good lens (f = 135 mm), an exposure of a few seconds is sufficient to obtain images of bands containing as little as 10 ng of DNA. With a long exposure time and a strong UV light source, the fluorescence emitted by as little as 1 ng of ethidium-bromide-stained DNA can be recorded on film. For detection of extremely faint DNA bands stained with this dye, a lens with a shorter focal length (f = 75 mm) should be used in combination with a conventional wet-process film (e.g., Kodak no. 4155). This setup allows the lens to be moved closer to the gel, concentrates the image on a smaller area of film, and allows for flexibility in developing and printing the image.

A further 10–20-fold increase in the sensitivity of conventional photography can be obtained by staining DNA with SYBR Gold (Molecular Probes). Of course, the price of this

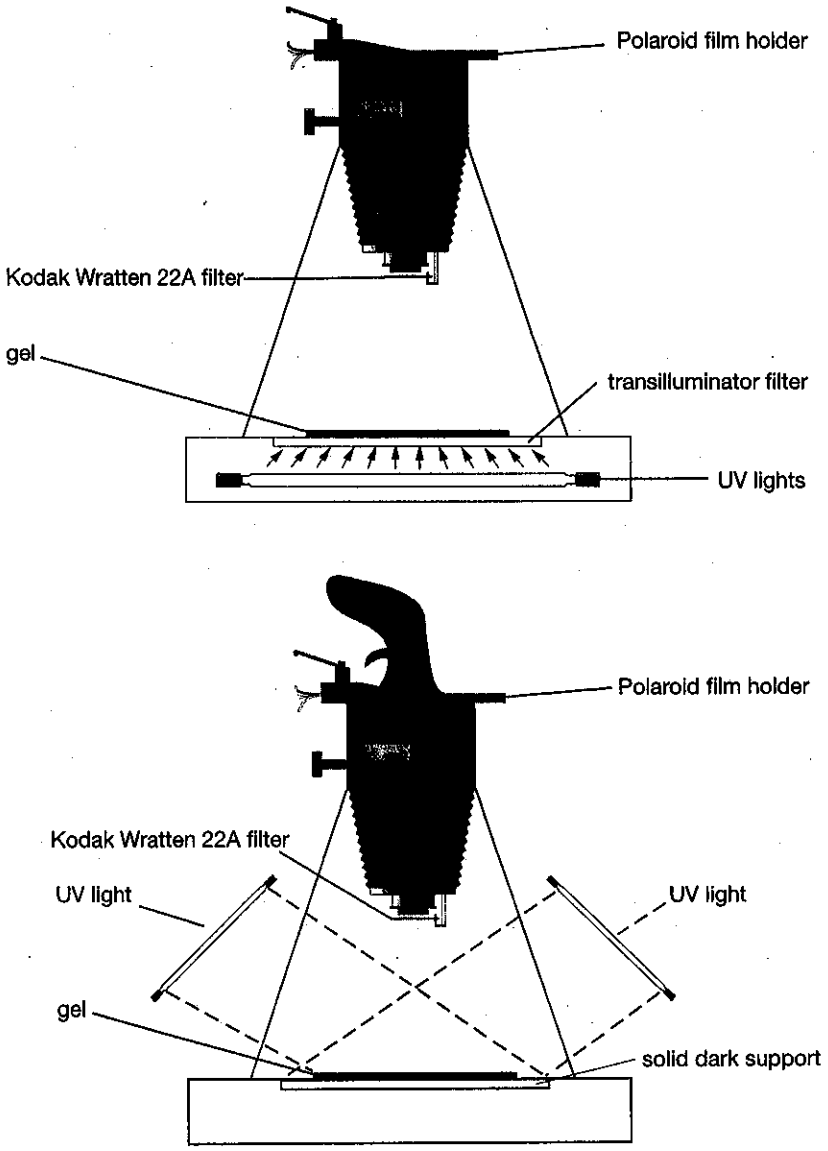

FIGURE 5-4 Photography of Gels by Ultraviolet Illumination

The top diagram shows the arrangement of the UV light source, the gel, and the camera that is used for photography by transmitted light. The bottom diagram shows the arrangement that is used for photography by incident light.

increase in sensitivity is steep: 10 liters of working solution of SYBR Gold stain costs more than 100 dollars, whereas the same amount of ethidium bromide costs ~5 cents. Detection of DNAs stained with this dye requires the use of a yellow or green gelatin or cellophane filter (S-7569, available from Molecular Probes or Kodak) with the camera and illumination with 300-nm UV light.