Gel Electrophoresis and Photography

An Application Note

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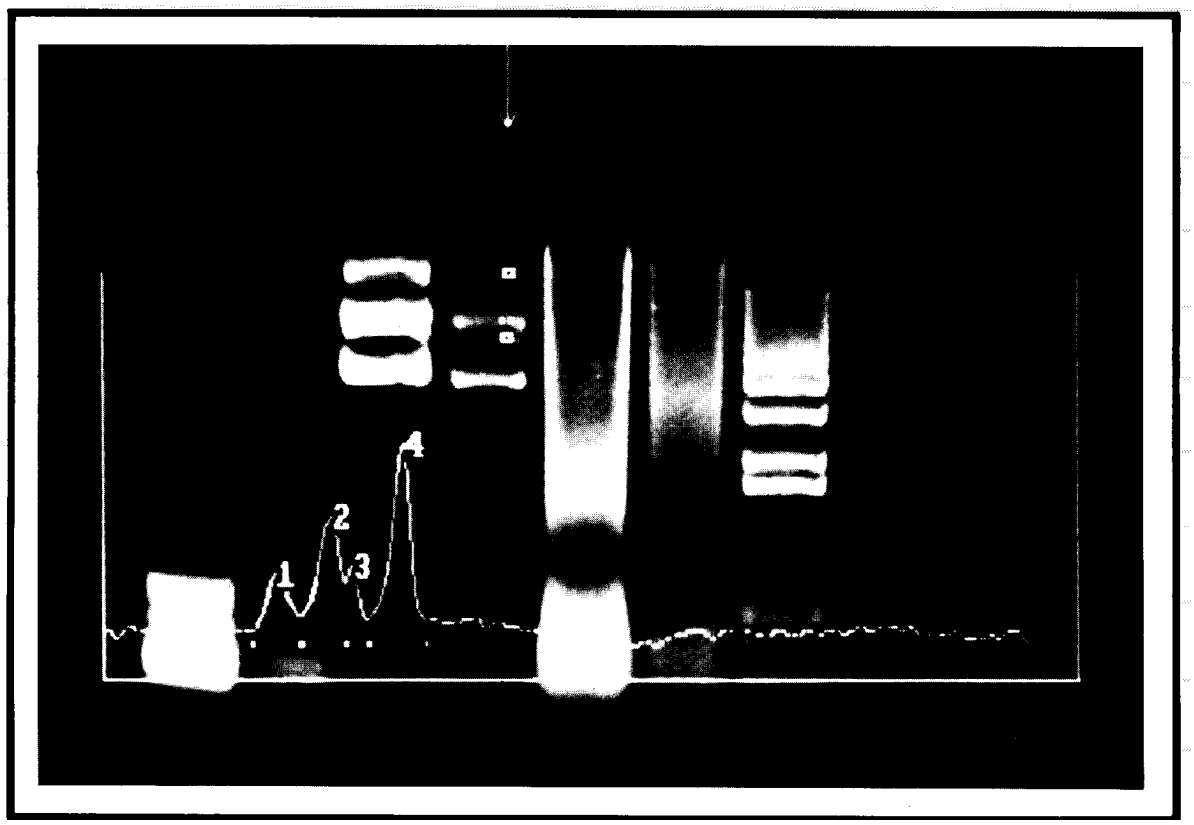


UVP

GEL ELECTROPHORESIS IMAGING, DOCUMENTATION AND ANALYSIS ... TODAY.

The GDS-8000 Gel Documentation Systems and software image analysis packages provide a comprehensive system for visualizing, analyzing and documenting ethidium bromide stained agarose and acrylamide gels, coomassie blue stained and silver stained protein gels, western blots, southern blots, dot blots, colony counting and autoradiographs. Powerful image acquisition software and hardware controls non-cooled or cooled CCD camera options. The C-80 Epi-Illumination Darkroom shown above features overhead UV for chemiluminescence and white light for a variety of applications. The pull-out drawer accepts UVP's Benchtop Transilluminators. The system is loaded with GelWorks or LabWorks software for advanced, intuitive analysis.

About the author

Dr. Simpson is a Professor in the Departments of Molecular, Cellular and Developmental Biology and Medical Microbiology, Immunology and Molecular Genetics, and an Investigator in the Howard Hughes Medical Institute at the University of California School of Medicine, Los Angeles. He is a member of the Molecular Biology Institute, the Jonsson Cancer Center and the Center for the Study of Evolution and the Origin of Life at UCLA, and a Foreign Member of the Brazilian Academy of Sciences. He did his undergraduate work in Biology at Princeton University and received a Ph.D. from the Rockefeller University in 1967 in Molecular Parasitology. After one year postdoctorate research at the Free University of Bruxelles in Belgium in the laboratory of Maurice Steinert, he assumed his current position at UCLA in 1969. His field of research is the molecular biology of trypanosomes, which are flagellated protozoa that cause several important human and animal diseases. His particular interest is the phenomenon of RNA editing that occurs within the Mitochondrion of trypanosomes, in which uridine residues are inserted and deleted at precise sites within mRNAs, thereby correcting frameshifts and sometimes creating entire genes. Dr. Simpson is also interested in the application of computers to the analysis of nucleic acid and protein sequences. His bibliography includes 141 scientific publications on this and related topics.

Cover Shot: A sample print produced from a GDS System and UVP's image analysis software of an ethidium bromide stained agarose gel with the bands identified on the overlayed scan.

The introduction of the technique of electrophoresis in acrylamide or agarose gels was a major advance in nucleic acid technology. Previously, large and expensive ultracentrifuge equipment was required to separate macromolecules on the basis of sedimentation coefficients Or buoyant densities, and scintillation counters were required to measure the radioactivity of the physically separated fractions. The advent of gel electrophoresis, ethidium bromide staining and autoradiography essentially brought an end to the era of intensive centrifugal analysis of nucleic acids. The development of these techniques was combined with the use of restriction enzymes and cloning in bacterial plasmids or phages to obtain large quantities of pure fragments of DNA molecules. Gel electrophoresis is simple, rapid, nondestructive and inexpensive, and, when combined with autoradiography, blot hybridization and elution of specific fragments from the gel, is an extremely powerful tool in the arsenal of recombinant DNA technology both for preparative and for analytical purposes.

Theory of Gel Electrophoresis:

If a molecule of net charge, q, is placed in an electric field, a force, F, is exerted upon it:

E = potential difference
q = net charge
d = distance between the positive and negative electrodes
E/d = field strength

This force is opposed by friction:

n = viscosity
v = velocity

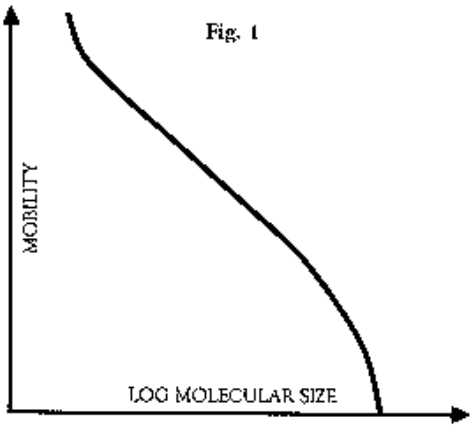
$$F = \frac{E}{d} q \quad \frac{E}{d} q = 6 \pi rnv \quad v = \frac{Eq}{do \pi rn}$$

However this equation is not adequate to explain electrophoresis in a gel, since it does not take into account the existence of gel pores. In reality, electrophoresis in a gel matrix can be thought of as a type of "gel filtration" where the driving force is the electric field. Penetration of the gel matrix by a mixture of nucleic acid molecules of different sizes results in the retardation of larger molecules. The electrophoretic mobility of nucleic acid molecules through a gel matrix is a function not only of molecular configuration (duplex or single stranded nucleic acid molecules), gel porosity, and ionic strength of the electrophoresis buffer.

Exclusion of nucleic acid macromolecules from the gel occurs if the average pore size is smaller than the size of the macromolecule. However in the case of duplex DNA, extremely large molecules can penetrate a gel by "snaking" through the pores in an extended fashion. This behavior gives rise to the nonlinear migration and pileup of large DNA molecules which is normally observed at the top of an agarose gel; this phenomenon has been used in the development of an elegant technique for the separation of extremely large duplex linear DNA molecules by field inversion and orthogonal field electrophoresis (FIGE and OFAGE). (Carle & Olson, 1984; Carle et al, 1986).

Standard gel electrophoresis is used to separate nucleic acid molecules ranging from oligonucleoticles to molecules approximately 20 kbp in size. Agarose, a nutural product derived from kelp, which melts at 100 C and solidifies at 42 C, is used for separation of molecules from approximately 100 bp to 20 kbp, and polyacrylamicle, an artificial polymer, is used for separation of small fragments. Different grades of agarose are available for different purposes: "low melting agarose" melts at 65 C and solidifies at 37^{o}C; "Nusieve" agarose (Marine Colloids Division of FMC Corp.) can be prepared at concentrations of 2-10% and can separate molecules down to 10 bp; "Ultrapure" agarose is used for preparative gel etectrophoresis and recovery of bands with fewer contaminants. Denaturing acrylamide gels can separate oligonucleotides differing by a single nucleoticle. Both double stranded and single stranded nucleic acids can be separated by electrophoresis. Conditions for denaturation usually involve high concentrations of urea and elevated running temperatures, formamide, methyl mercury, formaldehyde, or glyoxyol pretreatment.

The mobility of nucleic acid fragments in a gel matrix exhibits a complex but characteristic profile, as shown in Fig. 1.



Electrophoretic mobility of DNA or RNA in an agarose or acrylamide gel as a function of the size of the molecule. Note the regions of nonlinearity at the top and bottom of the gel and the central region of approximate linearity.

As mentioned above, large molecules which can not penetrate the gel pores in a normal fashion pile up at a short distance from the origin. Small molecules which migrate through the gel almost without retardation pile up in a smear at the bottom of the gel. Molecules of intermediate size migrate through the gel with a mobility approximately proportional to the log of their molecular size. This region of linearity varies with the gel concentration and must be empirically determined for each mixture of nucleic acid molecules for which molecular sizes are desired. The values presented in Table I can be used to select a gel for a desired separation. The marker dyes, bromphenol blue and xylene cyanol, are usually added to the sample prior to loading of the gel. The position of the marker dyes in the gel can be used to approximately determine the size of the nucleic acid fragments (Table 2). For accurate size determination, a mixture of fragments of known size is usually corun in one or more gel slots. Several algorithms are available for determination of the size of unknown fragments by least squares curve fitting procedures (Southern, 1979; Parker, 1980; Schaffer and Sederoff, 1981).

Table 1. Approximate gel concentrations for separation of DNA linear fragments of various sizes

Gel Concentration	Separation Range (bp)
agarose - 0.3%	60,000 - 5,000
agarose - 0.7%	20,000 - 800
agarose - 0.9%	7,000 - 500
agarose - 1.2%	6,000 - 400
agarose - 1.5%	4,000 - 200
agarose - 2.0%	3,000 - 100
agarose - 4.0%	500 - 10
acrylamide - 4%	1,000 - 800
acrylamide - 10%	500 - 25
acrylamide - 20%	50 - 1

Table 2. Size of DNA fragment that comigrates with marker dyes

Gel	Concentration	Bromphenol blue	Xylene cyanol
acrylamide	3.5%	100 bp	460 bp
	5	65	260
	8	45	160
	12	20	70
	20	12	45
acrylamide (denaturing)	5	35	130
	8	19	70
	10	12	55
	20	8	28

Detection of nucleic acids in gels:

Detection nucleic acids in agarose or acrylamide gels can be performed in several ways:

1. Staining with the intercalating dye, ethidiurn bromide, either after electrophoresis or during the run. Visualization of the DNA-dye complex bu UV illumination (Brunk and Simpson, 1977; Prumell, 1980). Method of choice for most purposes.
2. Staining of the gel with acridine orange and visualization of the nucleic acid-dye complex by UV illumination. Can distinguish single-stranded and double-stranded molecules (Carmichel and McMaster, 1980). Difficult to remove dye from gel-high background fluorescence.
3. UV shadowing. Illumination of the gel with a 254 nm UV source from above while the gel is resting on a thin layer silica gel plate with a fluorescent indicator dye incorporated into the silica gel (Hassur and Whitlocke, 1974). The DNA band appears as a dark, nonfluorescing band in a bright background. Excellent for recovery of oligonucleotides from acrylamide-urea gels.
4. Silver staining. This is the most sensitive method and provides a permanent record of the actual separation. A destructive method molecules can not be recovered from gel after staining.
5. The gel can be blotted onto a membrane filter (nitrocellulose or nylon) by capillary action or electrophoresis, and the blot either exposed to X Ray film (Kodak XAR-5) (if the nucleic acids are labeled) or hybridized with a labeled nucleic acid probe to detect sequence similarities. The blot can also be stained with a dye such as methylene blue to detect nucleic acid bands.

Table 3 presents the minimal detection limits for single-stranded and double-stranded DNA with several staining procedures.

Table 3. Minimum detection limits of single strand and double. strand DNA in acrylamide gels

	Detection Limit	
Stain	ssDNA (20% acrylamide 7 M urea)	dd DNA (5% acrylamide)
Ethidium bromide	0.25 ug	15 ng
Methylene blue	1.0 ug	300 ng
UV shadowing	2.0 ug	2 ng
Silver staining		2 ng

Practical Methods:

1. Gel Electrophoresis.

Agarose

Agarose gel electrophoresis is usually performed in a horizontal configuration with the gel submerged in the electrophoresis buffer ("submarine gel") (Fig.2). These units are relatively easy to construct from sheet lucite and are available commercially in several sizes. "Blitz" gels are made using large microscope slides (75 x 50 mm). These gels can be prepared in advance and stored covered with Saran Wrap at 4 C. Four or five samples (10 ul each) can be run rapidly (10-30 min) at high voltages (100-200 V) for rapid analysis of digestions or minipreps. Standard agarose gels are prepared on glass plates (13.5 x 20 cm) by pouring 200 ml of melted agarose cooled to 65 C. The agarose is melted in a microwave oven until no particles remain and the evaporated volume of water is added back to the solution to maintain the original ionic strength. Two basic types of electrophoresis buffers are used: Trisborate-EDTA (TBE = 90 mM Tris HCI, 20 mM boric acid, 2 mM EDTA, PH 8.3) and Tris-acetate (TAE = 40 mM Tris HCI, 20 mM Na acetate, 1.8 mM EDTA, PH 7.8). TAE is used for preparative separations when the gel is to be dissolved with Nal solutions prior to binding of the DNA with glass powder. TBE is used for most analytical work. However for separation of large DNA fragments, electrophoresis at a low voltage gradient in TAE buffer is optimal, due to the low extent of diffusion of large molecules. Denaturing buffers containing formaldehyde or methyl mercury are used for electrophoresis of single stranded nucleic acids.

For analytical purposes, 10-20 ul of sample are loaded per lane (5 mm). The sample buffer contains EDTA to stop the reaction, bromphenol blue and xylene cyanol, or Orange G to monitor the extent of electrophoresis, and glycerol or Ficoll to make the solution dense. Orange G has a higher mobility than bromphenol blue and xylene cyanol and causes less quenching of the ethidiurn bromide fluorescence. Ficoll yields sharper bands than glycerol, but the 10X stock solution is viscous and harder to pipet. To visualize the DNA by the ethidium bromide method, the dye (0.5 ug/ml) can be added to the electrophoresis buffer, or the gel can be stained for 30 min after the run.

Preparative agarose gels can be run using larger surface areas, thicker gels and larger sample wells. The maximum loading capacity of the gel is a function of the complexity of the fragment mixture and the average fragment size. Roughly, 50 ug/ ml of a complex mixture of fragments is the maximum that can be separated with high resolution.

Micropipetting samples for sequencing reactions. Samples are labeled with ^{32}P. A lucite shield and goggles protect the researcher from radioactivity.

Separation of extremely large (50 -2000 kbp) DNA molecules can be achieved in 1.0-1.5% agarose gels by the OFAGE or FIGE methods of Carle and Olson (1985) and Carle et al (1986). OFAGE relies on the fact that large DNA molecules subjected to periodic (10-120 sec) switching of orthogonal electric fields exhibit and "snaking" mobility in agarose gels that is a function of the molecular size. Smaller pulse times separate smaller molecules and longer pulse times, larger molecules. FIGE is a variation of this method in which the fields are 180 C apart; FIGE can be run in a standard submarine gel apparatus by merely inverting the field in a periodic manner. An increasing ramp of switching times is required to obtain adequate separation of a large range of fragment sizes, and this can be easily controlled by a microcomputer. A specialized sample preparation is also involved to eliminate breakage of large molecules by shear forces. Cells are embedded in low melting agarose and cell lysis is performed in blocks of agarose by diffusing in detergent and proteolytic enzyme. The blocks are then inserted into sample wells and the DNA electrophoresed into the agarose gel. The electrophoresis buffer is ½ TBE. Since much Joule heating occurs during the electrophoresis (24 hr at 250 V), the buffer is run through a heat exchanger to maintain the temperature at 13 C.

Acrylamide

Acrylamide gel electrophoresis is used to separate smaller fragments, double stranded or single stranded. The electrophoresis apparatus is vertical. TBE is the usual electrophoresis buffer. The gel is run without dye and is stained for 30 min after the run in 0.5 ug/ml ethidium bromide for visualization of bands. A denaturing buffer containing formamide or 7 M urea can be used to maintain molecules in a single stranded configuration. The width of the gel is 1-2 mm for most analytical purposes. Gradients of acrylamide can be used to produce sharp bands and increase the range of size resolutions. A glycerol density gradient is usually used to stabilize the acrylamide gradient during pouring of the gel.

2. Photography of ethidium-bromide stained gels.

Visualization of DNA (or RNA) bands with ethidium bromide is the method of choice for most purposes. Ethidium bromide (EB) (MW = 394.3) has a molar absorption coefficient at 486 nm of 4.83 X 10^3. The dye-DNA complex has an absorption maximum at 300 nm and a fluorescence emission centered at 590 nm. The fluorescence emission of the DNA-ethidium bromide complex is approximately 10-50X that of the uncomplexed dye. In addition to the emission at 590 nm from a stained gel, there is a major fluorescence emission peak

Micropipetting samples for restrition enzyme digestions prior to gel electrophoresis. The enzymes and samples are kep on ice to prevent degration.

at 450 nm due to the ethidium bromide-stained acrylamide or agarose gel alone. The available uv sources yield 254, 300 or 366 nm illumination. Transillumination from below is more efficient for visualization of weak bands, but epiillumination with hand held sources can be useful for monitoring of the extent of electrophoresis. UV-transparent plastic can be used for transillumination of stained gels with 300 and 366 nm uv, but not with 254 nm uv. The optimal detection of EB-stained DNA bands is accomplished by excitation of 300 run. The uv pass - visible block excitation filter used in the UVP T-15, T-36 and T-40 transilluminators transmits 300 nm uv with about 70% efficiency, but also allows some visible red light to pass.

The size of the exciter filter is an important consideration in purchase of a transilluminator. The 21.5 x 40 cm size of the UVP model T-40 accommodates two 20 x 20 cm gels at once or a single large acrylamide gel 20.5 x 39 cm).

The extent of uv-induced DNA damage is another consideration in the choice of a visualization method. The 254 nm source is vastly more damaging in terms of photonicking and photodimerization than the 300-nm or the 366-nm sources. Photobleaching, probably due to uv-induced dissociation of the DNA-dye complex, is appreciable with the 254-nm source and very slight with the 300 and 366-nm sources. Less DNA damage occurs with the 366-nm source than with the 300-nm source, but the sensitivity for detection of the DNA-dye complex is only about 1/10 of the 300 nm source. The 300-nm source can be used for preparative as well as analytical purposes provided photography is performed rapidly. Focusing of the camera should be done prior to turning on the uv source. One should not leave the gel being exposed to the transilluminator while examining the patterns. Use of a 366 nm uv source is indicated when no damage to the DNA can be tolerated in a preparative experiment, but the loss in sensitivity almost outweighs the benefits gained. The 300-nm uv source is a good compromise in terms of limited photodamage to the DNA and high sensitivity of visualization.

Photography of the stained gel is performed using a barrier filter consisting of a Wratten #22 filter sandwiched between window glass, camera setup such as the Polaroid MP-4 and a 75 mm lens is ideal for photography. Polaroid photographs are taken using type 667 film in a 44-48 pack film holder or type 57 single sheet film. Negatives are taken using 4 x 5 inch Kodak Royal Pan X sheet film in a single sheet holder that replaces the Polaroid pack film holder. Exposures are 1-2 sec for the Polaroid film and 5-10 sec for the Royal Pan film at f-8. These exposures times with the 300 nm uv source should cause little if any damage to the DNA in the gel.

A transparent plastic ruler lying on top of the gel at one side is always included in the photograph to provide an absolute scale for determining the position of autoradiographic bands after blot hybridization.

Destaining of the gel is only useful when the fragments are very large and do not readily diffuse, such as in the case of OFAGE (Carle and Olson, 1984) or FIGE (Carle et al, 1986)

separation of chromosomes. Overnight destaining in water at 4 C improves the resolution dramatically in this case.

A standard mixture of known DNA fragments at a known concentration should be included in one or two lanes of every gel (Table 4S. This provides not only a molecular weight calibration, but also a densitometric reference for estimating the amount of DNA in unknown bands (Prunell, 1980).

Calculation of molecular weights of unknown bands can be performed using several least squares curve fitting algorithms. Usually several methods are used for the standard curves, such as the methods of Southern (1979), Parker (1980) and Schaffer and Sederoff (1980), and the deviations of individual bands compared. A deviation of more than 50/6 from the calculated curve is not acceptable. The methods usually agree nicely in the "linear" region of the gel, but disagree significantly in the nonlinear regions. It should be noted that certain DNA sequences can cause significant deviations (retardation) from the expected migration behavior in acrylamide gels (but not in agarose gels) due to conformational "bending" of the duplex helix (Marini et al, 1982; Kidane et al, 1984).

Table 4. Size of several reference DNA fragments.

	HaeIII		HindIll	
1-	1,353	bp	23,130	bp
2-	1,078		9,416	
3	872		6,557	
4-	603		4,361	
5-	310		2,322	
6a-	281		2,027	
6b-	271		564	
7	234		125	
8-	194			
9-	118			
10-	72			

3. Elution of bands from gels and recovery of samples.

Elution of nucleic acid molecules from agarose and acrylamide gels can be accomplished with variable yields depending on the molecular size of the molecules. This, however, is the least satisfactory aspect of gel electrophoresis, since yields are never quantitative and contaminants often elute together with the nucleic acids. Small DNA and RNA molecules (10- 1000 bp) can be eluted with good yields by simple diffusion; larger molecules (1000 bp -10,000 bp) can be electoeluted with yields ranging from 10-80%. Often, especially with agarose, contaminants coelute with the nucleic acids which may interfere with subsequent enzymatic steps. Quantitative elution of nucleic acids from gel bands represents an unsolved problem, for which, however, there are a considerable number of partial solutions which suffice for most purposes. Methods useful for cleaning gel-eluted DNA include the Nensorb (New England Nuclean Co.) and Elutip (Schleicher and Schuell) disposable columns, and the Geneclean (Bio 101) powdered glass absorption technique.

a. Several Selected Methods for Elution of DNA from Gels:

a. Electroelution into dialysis bags. The bands are excised from the gel using a plastic knife to prevent damage to the uv filter, and cut into small pieces, which are inserted into a dialysis bag together with 3 ml of 1/10 TBE buffer. The bag is covered with TBE buffer and subjected to electrophoresis for 1-3 hr with cooling at 200 ma. The supernatant solution is removed and the elution repeated once until no more DNA is left in the gel as visualized by uv transillumination. The pooled supernates are dialyzed and the DNA precipitated with salt and ethanol.

b. Electroelution onto NA45 DEAE nitrocellulose filters. The filter is inserted into a slit in front of the band and the band electrophoresed into the filter. The filter is removed and cut into small pieces and the DAn eluted with a high salt solution.

c. Binding of DNA on glass powder. "Geneclean" (Bio 101 Co.) is a commercial preparation of finely powdered glass which binds duplex DNA. The DNA bands from a TAE-agarose gel are excised and the agarose dissolved in saturated NAI solution. Glass powder is added to the solution to bind the DNA. DNA is eluted from the washed glass powder with a dilute buffer or water.

4. Blotting and hybridization of gels.

Agarose gels can be blotted onto nitrocellulose or nylon filters either by capillary action by electrophoresis. Pretreatment of the gel with 0.25 N HCl for 30 min prior to denaturation and blotting results in a decrease in the average molecular weight of all DNA fragments by limited depurination and thereby an increased rate of diffusion onto the filter. The simplest blotting method is the original method of Southern (1979) and the modified "diblot" method of Smith and Summers (1980), in which the gel is blotted simultaneously onto two filters (or unidirectionally onto one filter) by using the liquid within the gel itself as the source. For complete transfer, paper wicks are used to continuously draw liquid from two reservoirs through the gel and the adjacent filter. Multiple blots of separate lanes can be performed together with a single filter provided the gel slices are slightly separated so that the filter can be cut into separate lanes after the blot. The blot should be marked with a ball point pen to indicate the orientation and position of the gel origin. Nitrocellose filters of 0.22 um porosity retain smaller DNA fragments than filters of 0.45 um porosity. Nylon filters are stronger and easier to handle than nitrocellose and allow transfer to be performed even with low ionic strength buffers (e.g. for electroblotting), or with NaOH so as to avoid the denaturation step in the blotting process. However nylon filters yield a higher background level than nitrocellulose, which must be countered with the use of higher concentrations of blocking agents such as SDS and Denhardt's solution. DNA remains attached to nylon filters and the filters can be reprobed multiple times, unlike nitrocellulose which can only go through two or three rehybridizations before the DNA is lost from the filter.

Acrylamide gels can be electroblotted onto nitrocellulose or nylon filters in c itrate -phosphate buffer at pH 3.0 (Smith et al, 1984).

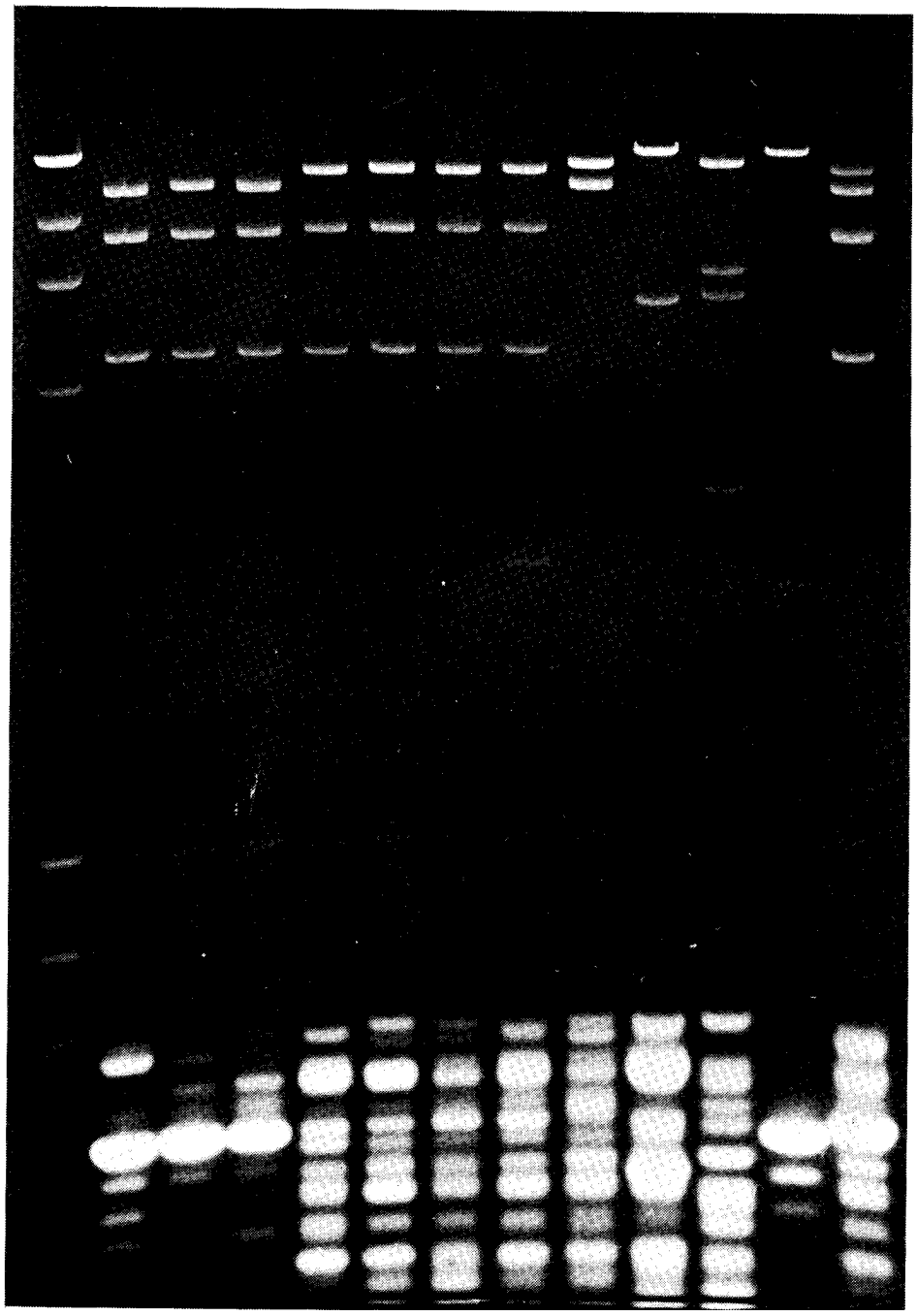
Hybridization is conveniently performed in "Seal-a-Meal" bags. Washing of filters is performed in plastic trays floating in a water bath. Exposure of hybridized blots is carried out at room temperature or at -70 C if prefogged film and an intensifying screen is used to avoid reciprocity loss. Several different exposures should be obtained so as to avoid nonlinear exposure of major bands and to visualize minor bands.

The stringency of hybridization is controlled by varying the temperature and salt concentration both of hybridization and washing and by varying the percentage of formamide in the hybridization medium. The following equation can be used to calculate the TM for a DNA fragment of known base composition:

$$TM = 81.5 + 16.6 \log M + 0.41 (\%GC) - 0.72 (\% \text{ formamide})$$

M = monovalent salt concentration (Howley et al, 1979)

0.8% agarose gel in Tris-borate-EDTA buffer with 0.5 ug/ml ethidium bromide of kinetoplast DNA from several lizard *Leishmania* strains and species digested with the HaeIII restriction enclonuclease. The leftmost lane contains a mixture of lambda DNA digested with HindIII and bacteriophage OXI 74DNA digested with HaellI to serve as size markers. The upper bands in the kinetoplast DNA digests represent fragments of the maxicircle DNA



and the intensely stained lower bands represent fragments of the minicircle DNA.

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