

which usually takes less than an hour. The database should be monitored for random distribution of sequences; accumulation of specific sequences, which indicates nonrandom sonication or some cloning problem, will become apparent early on. In practice, however, sonication seems to produce random libraries from DNAs varying widely in base composition and from a variety of organisms. Some minor editing may be necessary during data accumulation, but it is most efficient to collect the bulk of the sequence data prior to editing. The redundancy of data at this stage of the project makes it easier to resolve problem regions.

The development of a semiautomated microtiter protocol for template preparation overcomes a rate-limiting step in large sequencing projects. The current limitation in the procedure described, using the Applied Biosystems 373A, is gel throughput. However, this method offers significant advantages. In particular, the manual digitizing of autoradiographic images is no longer necessary and we consistently obtain greater than 400 nucleotides of accurate sequence per clone. The combination of this base-calling capability with high-throughput microtiter methods for template preparation and sequencing reactions is an effective implementation of the shotgun strategy.

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[14] DNA Sequencing with Direct Transfer Electrophoresis and Nonradioactive Detection

By PETER RICHTERICH and GEORGE M. CHURCH

Introduction

In DNA sequencing by base-specific chemical degradation¹ or chain termination,² two steps that are critical to the overall efficiency and conve-

¹ F. Sanger, S. Nicklen, and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).

² A. M. Maxam and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 560 (1977).

nience of the procedure are the separation of the DNA fragments by polyacrylamide gel electrophoresis and the subsequent visualization of the sequencing patterns. Increasing the number of readable nucleotides per sequencing reaction reduces the number of template DNA preparations and sequencing reactions; furthermore, the sequence assembly from individual reactions can be significantly simplified. To maximize the number of readable bases, very thin gels, very long gels, low acrylamide concentrations, and multiple loadings have been used.³⁻⁶

One limiting factor in conventional gel electrophoresis is the unequal spacing of bands over the length of the gel. This results from the inverse relationship between DNA size and electrophoretic mobility. Lowering the electrical field toward the bottom of the gel by employing buffer or thickness gradient gels⁷⁻⁹ reduces this effect, but does not eliminate it completely.

Constant spacing between bands can be obtained by a different observation mode: instead of looking at the whole gel at a fixed point in time, a fixed point (or line) in the gel is observed over a prolonged time period. Examples of separation methods that employ such "spatially fixed" detection are preparative electrophoresis methods and direct transfer electrophoresis.¹⁰ In direct transfer electrophoresis, a membrane that is moved along the bottom of the gel immobilizes the DNA as it elutes out of the gel. This transforms the temporal order of elution into a spatial one on the membrane. Because the detection (or rather elution) time depends linearly on the size of the DNA fragments, a constant membrane transfer speed yields equal interband distances. The original description of direct transfer electrophoresis for DNA sequencing¹⁰ showed the potential benefits of this method; however, the band patterns that were generated with 14-cm long, 0.35-mm thick gels were insufficiently resolved and made reliable sequence interpretation difficult. The subsequent use of thinner and longer gels allowed for the resolution of more than 350 nucleotides per lane set.¹¹ Here, we describe optimizations of apparatus design and gel parameters

³ F. Sanger and A. R. Coulson, *FEBS Lett.* **87**, 107 (1978).

⁴ W. Ansorge and L. deMayer, *J. Chromatogr.* **202**, 45 (1980).

⁵ D. R. Smith and J. M. Calvo, *Nucleic Acids Res.* **10**, 2255 (1980).

⁶ W. Ansorge and R. Barker, *J. Biochem. Biophys. Methods* **9**, 33 (1984).

⁷ M. D. Biggin, T. J. Gibson, and G. F. Hong, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3963 (1983).

⁸ W. Ansorge and S. Labeit, *J. Biochem. Biophys. Methods* **10**, 237 (1984).

⁹ A. Olsson, T. Moks, M. Uhlén, and A. B. Gaal, *J. Biochem. Biophys. Methods* **10**, 83 (1984).

¹⁰ S. Beck and F. M. Pohl, *EMBO J.* **3**, 2905 (1984).

¹¹ P. Richterich, C. Heller, H. Wurst, and F. M. Pohl, *BioTechniques* **7**, 52 (1989).

that now allow us to resolve fragments up to more than 800 nucleotides long.

While the membrane-bound sequence patterns produced by direct transfer electrophoresis can be detected by conventional radioactive labeling, they also offer a starting point for other detection methods that require a membrane-bound pattern, such as genomic sequencing,¹² multiplex sequencing,¹³ or sequencing with enzyme-linked detection.¹⁴ The use of nonradioactive labeling and detection methods offers a number of potential advantages; these include reduced costs, fewer health and regulatory risks, increased efficiency, and longer reagent shelf life.

DNA sequencing requires a high detection sensitivity because a typical band contains about 0.02–1 fmol of DNA (12 million to 600 million molecules). These requirements have been met by radioactive,^{1,2} fluorescent,^{15–18} and enzyme-linked detection systems.^{14,19–21} Enzyme-linked detection offers advantages for both high throughput and occasional needs. For high throughput, automatic film readers^{22,23} and development automations¹¹ can be considerably faster and less costly than fluorescent devices. For occasional sequencing, the higher stability of reagents and sequencing reaction products of nonradioactive systems can be helpful; again, these advantages can be achieved with significantly lower equipment costs for enzyme-linked detection systems than with fluorescent devices.

For DNA sequencing with enzymatic detection, oligonucleotide primers end labeled with biotin¹⁴ or digoxigenin²⁴ have been used. A good signal-to-noise ratio is critical for the performance of the enzymatic detection method; good results using the biotin-based system have been ob-

¹² G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984).

¹³ G. M. Church and S. Kieffer-Higgins, *Science* **240**, 185 (1988).

¹⁴ S. Beck, *Anal. Biochem.* **164**, 514 (1987).

¹⁵ L. M. Smith, J. Z. Sandes, R. J. Kaiser, P. Hughes, C. Dodd, C. R. Connell, C. Heiner, S. B. H. Kent, and L. E. Hood, *Nature (London)* **321**, 674 (1986).

¹⁶ J. M. Prober, G. L. Trainor, R. J. Dam, F. W. Hobbs, C. W. Robertson, R. J. Zagursky, A. J. Cocuzza, M. A. Jensen, and K. Baumeister, *Science* **238**, 336 (1987).

¹⁷ W. Ansorge, B. Sproat, J. Stegemann, C. Schwager, and M. Zenke, *Nucleic Acids Res.* **15**, 4593 (1987).

¹⁸ J. A. Brumbaugh, L. R. Middendorf, D. L. Grone, and J. L. Ruth, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5610 (1988).

¹⁹ P. Richterich, *Nucleic Acids Res.* **17**, 2181 (1989).

²⁰ S. Beck, T. O'Keeffe, J. M. Coull, and H. Köster, *Nucleic Acids Res.* **17**, 5115 (1989).

²¹ R. Tizard, R. L. Cate, K. L. Ramachandran, M. Wusk, J. C. Voyta, O. J. Murphy, and I. Bronstein, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4514 (1990).

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²³ M. J. Eby, *Bio/Technology* **8**, 1046 (1990).

²⁴ F. M. Pohl, unpublished results, 1990.

tained by using streptavidin and biotinylated phosphatase. Nonspecific binding of streptavidin is efficiently minimized by high concentrations of sodium dodecyl sulfate (SDS) in the incubation buffer,¹¹ and nonspecific binding of biotinylated alkaline phosphatase is easily blocked by several detergents or proteins. In the digoxigenin system, milk protein fractions work effectively as blocking agents.²⁵

One significant advantage of nonradioactive labels is the possibility of combining several different labels in one experiment, an approach that might be called "label multiplexing." The use of different primer oligonucleotides labeled with different fluorophores in fluorescent sequencing machines^{15,16} can be seen as one example. Another example is the use of two hybridization probes labeled with different enzymes, alkaline phosphatase and peroxidase, for nucleic acid hybridizations.²⁶ We describe the simultaneous use of two different labels, biotin²⁷ and digoxigenin,²⁵ for enzymatically detected DNA sequencing. This procedure doubles the amount of sequence information that can be obtained from a gel.

We also compare different commercially available chemiluminescent enzyme-substrate systems: peroxidase-based chemiluminescence with luminol and enhancers,²⁸⁻³⁰ and two chemiluminescent dioxetane substrates for alkaline phosphatase.³¹⁻³³ A protocol is described for combining peroxidase-based enhanced chemiluminescence detection of digoxigenin-labeled primers and phosphatase-based chemiluminescence detection of biotinylated primers to generate two sequence patterns from one gel in a total time of less than 3 hr.

Future extensions of this approach toward the use of more labels, thus going from "label duplexing" to "label multiplexing," may require the repeated use of one enzyme with different labeling systems. Such a proto-

²⁵ C. Kessler, H.-J. Hölte, R. Seibl, J. Burg, and K. Mühlegger, *Biol. Chem. Hoppe-Seyler* **371**, 917 (1990).

²⁶ M. Renz and C. Kurz, *Nucleic Acids Res.* **12**, 3435 (1984).

²⁷ J. J. Leary, D. J. Brigati, and D. C. Ward, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4045 (1983).

²⁸ T. P. Whitehead, G. H. G. Thorpe, T. J. N. Carter, C. Groucutt, and L. J. Kricka, *Nature (London)* **305**, 158 (1983).

²⁹ G. H. G. Thorpe, L. J. Kricka, E. Gillespie, S. Mosely, R. Amess, N. Baggett, and T. P. Whitehead, *Anal. Biochem.* **145**, 96 (1985).

³⁰ I. Durrant, L. C. A. Bengel, C. Sturrock, A. T. Devenish, R. Howe, S. Roe, M. Moore, G. Scozzafava, L. M. F. Proudfoot, T. C. Richardson, and K. G. McFarthing, *BioTechniques* **8**, 564 (1990).

³¹ A. P. Schaap, M. D. Sandison, and R. S. Handley, *Tetrahedron Lett.* **28**, 1159 (1987).

³² I. Bronstein, J. C. Voyta, and B. Edwards, *Anal. Biochem.* **180**, 95 (1989).

³³ I. Bronstein, R. R. Juo, Voyta, and B. Edwards, in "Bioluminescence and Chemiluminescence: Current Status" (P. E. Stanley and L. J. Kricka, eds.), p. 73. Wiley, Chichester, England, 1991.

col would necessitate the removal or inactivation of the enzyme between sequential detection steps. We show the feasibility of sequential detections using differently labeled primers with phosphatase-based chemiluminescence.

Principle of Direct Transfer Electrophoresis

In direct transfer electrophoresis (DTE), a membrane is moved along the lower surface of the gel; molecules that elute from the gel and move toward the lower electrode are immobilized on the membrane. The membrane is usually an unmodified or positively charged nylon membrane, but other materials, such as nitrocellulose, have also been used.^{10,34} Most apparatus designs use a conveyor belt made of nylon or polyester mesh to transport the membrane. The conveyor belt is moved by a stepper motor that is connected to axles with timing pulleys and a timing belt. The stepper motor is controlled via electronic timing circuits or, more conveniently, by a small computer. The conveyor belt is usually moved with constant speed (see Introduction), but different speed gradients or different speeds can also be used.¹⁰

Because the spacing of bands on the membrane can be controlled by the transport speed in direct transfer electrophoresis, gels as short as 14–20 cm can be used for DNA sequencing; they offer a convenient alternative to normal long sequencing gels. When extended reads and higher resolution, especially for longer fragments, are the objectives, longer DTE gels can give superior results (see Discussion).

Materials

Enzymes, Plasticware, Machines

Anti-digoxigenin–phosphatase conjugates (Fab fragments) and anti-digoxigenin–peroxidase conjugates (Fab fragments) (Boehringer Mannheim, Indianapolis, IN)

Biotinylated alkaline phosphatase: Purchase from Molecular Probes (Eugene, OR) and Millipore/New England BioLabs (Beverly, MA) or prepare as described;¹¹ note that after biotinylation, two subsequent purifications are essential to obtain a maximal signal intensity, and that lyophilization or freezing of the biotinylated phosphatase leads to increased background and largely reduced signal intensities

³⁴ F. M. Pohl and S. Beck, this series, Vol. 155, p. 250.

- Modified T7 DNA polymerase (Sequenase): Purchase from United States Biochemicals (Cleveland, OH)
- Polyester plastic material of various thicknesses: Purchase from AIN Plastics and LKB (Rockville, MD)
- Thin shark's-tooth combs (0.125 mm) with 2.25-, 3.0-, and 4.5-mm wide wells (Owl Scientific)
- Twelve-channel syringe pipettors (Cat. No. 0155200; Hamilton, Reno, NV)
- Direct transfer electrophoresis machines and large Plexiglas trays for nonradioactive developments: Ours were made in the Harvard Medical School machine shop.
- Portable computers (Tandy 102): Used as pulse generators for the stepper motor
- Power supply (6000 V) (Fisher Scientific, Pittsburgh, PA)
- Programmable, tilt-adjustable roller for four bottles (14-cm i.d., 38 cm long for wide membranes): From BioComp (Fredericton, New Brunswick, Canada)

Chemicals

The sources we have used are given below; other chemicals from other suppliers may be adequate.

- 3-(Trimethoxysilyl)propyl methacrylate: Aldrich (Milwaukee, WI)
- BCIP (5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt) and NBT (Nitro Blue Tetrazolium): Sigma (St. Louis, MO) and GIBCO/Bethesda Research Laboratories (Gaithersburg, MD)
- Biotin-XX-NHS ester: Clontech (Palo Alto, CA)
- Chemiluminescent dioxetane substrates for alkaline phosphatase {disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2'-tricyclo[3.3.1.1.^{3,7}]decan-4-yl)phenyl phosphate]}: Called AMPPD (Tropix, Bedford, MA) or LumigenPPD (Millipore/New England BioLabs)
- AMPPD-Cl (5-chloro-substituted adamantyl dioxetane): Tropix
- Chemiluminescent substrates for horseradish peroxidase: ECL gene detection system (Amersham, Arlington Heights, IL)
- Diethanolamine: Sigma (ACS reagent) and Tropix
- NHS-digoxigenin (digoxigenin-3-*O*-methylcarbonyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide ester), blocking reagent on milk protein basis: Boehringer Mannheim
- Nylon membrane: Biotodyne A, 1.2- μ m pore size, BNNG3R: Pall (Glen Cove, NY)
- Sephadex G-25 columns (NAP-5 and NPA-10): Pharmacia (Piscataway, NJ)

Streptavidin: Sigma, Millipore/New England BioLabs, and others
Stains-all, Tris (Trizma base), Tris-HCl (Trizma hydrochloride), and
Triton X-100: Sigma
Triethylamine acetate, pH 7.0 (2 M solution): Applied Biosystems (Foster
City, CA)

Solutions and Buffers

For oligonucleotide labeling

NaCO₃ (1 M), pH 9.0

Stains-all (20×): 1 mg/ml Stains-all in formamide; store in a dark bottle
(Stains-all is 1-ethyl-2-[3-(1-ethylnaphthol[1,2-*d*]thiazolin-2-ylidene)-
2-methylpropenylnaphthol[1,2-*d*]thiazolium bromide)

Stains-all (1×): Dilute 20× Stains-all in 50% formamide shortly before
use

For dideoxy sequencing

Dithiothreitol (DDT) (0.1 M)

Annealing buffer (6.5×), 1 ml: 27.4 μl 1 M MnCl₂, 65.5 μl 1 M trisodium
isocitrate, 274 μl 1 M Tris-HCl, pH 7.5 (at 25°), 40 μl 5 M NaCl, 590
μl H₂O; store in screw cap vial at -20°, replace when dark brown
TE: 10 mM Tris-HCl (pH 8.0), 0.1 mM ethylenediaminetetraacetic acid
(EDTA)

Nucleotide mixes: dATP, dCTP, dGTP, and dTTP (250 μM each) and
the respective ddNTP (1 μM) in water; store at -20°

Formamide-dye mix: 95% (w/v) deionized formamide, 5 mM
Na₂EDTA, pH 8, 0.04% (w/v) bromphenol blue, 0.04% (w/v) xylene
cyanol FF; store at -20°

*For gel electrophoresis*³⁴

Bind silane dilution: 0.5% (v/v) 3-(Trimethoxysilyl)propyl methacrylate,
0.3% (v/v) acetic acid, 3% (v/v) water, 96.2% (v/v) ethanol; store dark
at 4°

TBE (10×): 154.5 g Tris base, 26.2 g boric acid, 9 g Na₂EDTA, 810 ml
H₂O

Urea buffer: 486.5 g H₂O g urea, 93.5 g 10× TBE; store at room tempera-
ture for up to 2 months

Ammonium persulfate (10%, w/w): Prepare fresh before use or store
aliquots at -20°

Acrylamide solution [3.5% (6%), w/v]: 3.4 g (5.7 g) acrylamide, 0.1 g
(0.3 g) bisacrylamide, 97 (95) ml urea buffer; filter, use within 24 hr:
degas before adding *N,N,N',N'*-tetramethylethylenediamine
(TEMED) and ammonium persulfate

For nonradioactive detection

MgCl₂ (1 M), sterilized

SDS, 20%

Triton buffer (10×): 44.4 g Tris-HCl (Sigma), 26.5 g Tris base, 73 g NaCl, 3.7 g Na₂EDTA, 818 ml water; dissolve, add 100 ml Triton X-100; store at 4° for up to 4 weeks

Triton buffer (1×): Dilute 10× Triton buffer to 1× with distilled water, and filter; use within 24 hr

SDS buffer: 100 ml 10× Triton buffer, 250 ml 20% (w/v) SDS, 650 ml H₂O; filter, use within 24 hr

Block buffer (for digoxigenin-based detections): Dissolve 10 g of blocking reagent (purified casein fractions; Boehringer Mannheim) in 1 liter 0.1 M Tris-HCl (pH 7.6), 150 mM NaCl by stirring at 55–65° for 1 hr

DEA buffer (10×): 885 ml water, 32.2 g Tris-HCl, 105.2 g diethanolamine (store the diethanolamine at 37°); store at 4°

DEA buffer (1×): Dilute 10× DEA buffer to 1× with distilled water, add 1/1000 vol of 1 M MgCl₂, and filter; use within 24 hr

Dioxetane substrate solution: 0.1 mg/ml AMPPD (or AMPPD-Cl) in 1× DEA buffer with 50 mM MgCl₂; prepare immediately before use

Tris-HCl (10×), pH 9.5: 4.33 g Tris-HCl, 87.5 g Tris base, 43.9 g NaCl, 9.68 g MgCl₂ · 6H₂O; use within 24 hr

BCIP stock solution: 50 mg/ml in dimethylformamide (DMF); store at –20° (only the *p*-toluidine salt of BCIP will dissolve in DMF)

NBT stock solution: 75 mg/ml in 70% (v/v) dimethylformamide; store at –20°

BCIP/NBT substrate solution: Add 33 μl NBT stock solution and 33 μl BCIP stock solution per 10 ml 1× Tris-HCl, pH 9.5; mix after each addition; prepare shortly before use

Stripping buffer: 1% (w/v) SDS, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), 125 mM NaCl

Direct Transfer Electrophoresis Protocols*Apparatus Design*

To obtain the best transfer results, a constantly good contact between the lower gel surface and the membrane is essential. Several concepts have been used previously to achieve this goal, for example, brakes on the rear axle,³⁴ tensioning motors on front and rear axle, and metal weight cylinders on the conveyor belt.¹¹ A novel method is used in the machine

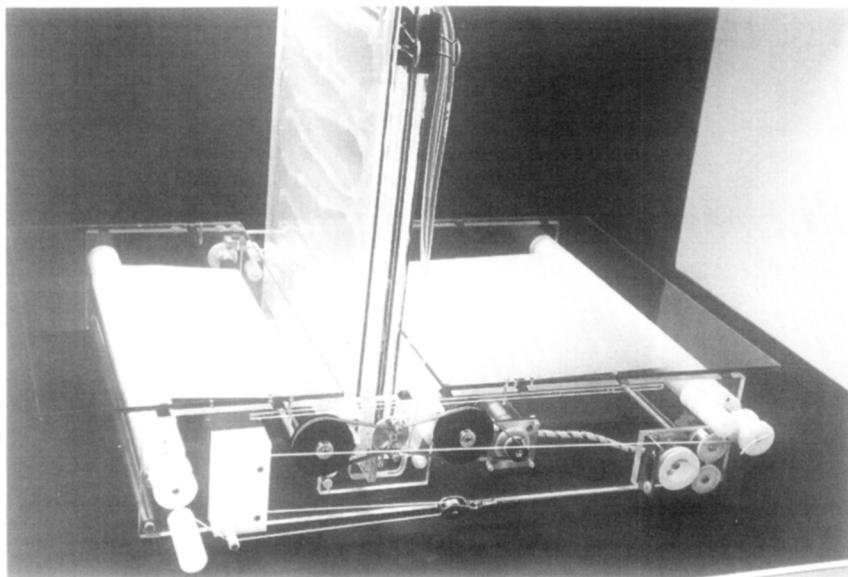


FIG. 1. An apparatus for direct transfer electrophoresis. The conveyor belt is moved from left to right by a stepper motor that is connected to two axles via a timing belt and timing pulleys. A string that is tensioned by a spring motor (front right) via a pulley connects front and rear axle. The outer dimensions of the machine are $55 \times 70 \times 13$ cm.

shown in Fig. 1: a string pulls on both the front and the rear axle of the machine; the string is tensioned by a spring motor via a pulley. By pulling from both ends, any slack of the conveyor belt that might build up during the run is effectively removed. The spring motor exerts similar levels of force, regardless of its extension. Therefore, the system can take up a considerable amount of lengthening (or shortening) of the conveyor belt, providing superior performance and reliability over long periods of use.

Glass Plate and Thermostatting Design

One point that needs special attention in direct transfer electrophoresis is the lower surface of the gel itself: the gel polymerization must be uniform at the bottom, the glass plates must be flat over the whole width of the gel, and they need to be aligned carefully. Even polymerization can be achieved by sealing the gel bottom with Parafilm and pressing it against a vertical surface¹¹ as described below. The lower edges of glass plates usually show slight curvature after being cut, often an inverted U shape; when such plates are used, contact in the middle of the gel can be insuffi-

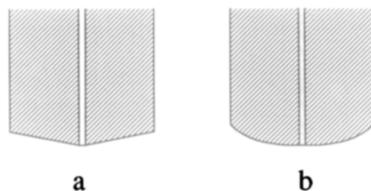


FIG. 2. Schematic drawing of the polished bottom edge of glass plates for DTE. (a) Side view of machine-polished plates; (b) manual polishing gives a rounded shape that makes it easier to determine if the plates are perfectly aligned.

cient. Machine-polished glass plates with an angle of 5–15° at the bottom as shown in Fig. 2a offer better performance and can be obtained by precision glass suppliers. Alternatively, plates can be manually polished to the shape shown in Fig. 2b. Because the rounded shape makes it easier to feel if the plates are correctly aligned, we usually subject even machine-polished plates to a short manual fine-polishing.

Manual Polishing of Glass Plates. Use freshly cut glass plates with unseamed bottom surfaces. Clean and align a set of plates; especially with 40-cm wide glass plates, check that they have either no curvature or identical curvature over their width. If the curvature differs slightly, the thickness of the gel will differ from the side toward the middle. When such plates are used, it can be difficult to insert the spacer, the shark's-tooth combs may leak, or bands in the middle of the gel may be wider than on the sides.

Clamp the plates together and polish them on wet, waterproof sanding paper, fixed to a flat bench with adhesive tape; use long stretches of sanding paper, starting with coarse (grit 120–220) paper. Try to obtain a shape as shown in Fig. 2b. Switch to finer sanding paper (320, finally 400) when they show an evenly rounded, well-aligned surface.

Glass Plate Setup for Thermostatted Gels. Although aluminum plates on one side of the gel assembly can be used to eliminate smiling, they can produce electrical short circuits, especially when voltages above 2000 V are used. Upper buffer chambers that extend almost over the whole length of the gel offer a convenient alternative. They can be easily made with glass spacers and silicone rubber adhesive.

To maintain a constant temperature independent from the applied voltage and the room temperature, we use a simple thermostating design. Plastic tubing (Tygon, o.d. 6 mm, wall thickness 0.8 mm; VWR Scientific, South Plainfield, NJ) is mounted onto a plastic sheet with cable ties and connected to a pumping water bath (Model 1130, VWR Scientific) through quick coupling connectors (see Fig. 1). The glass plate, spacer, and buffer

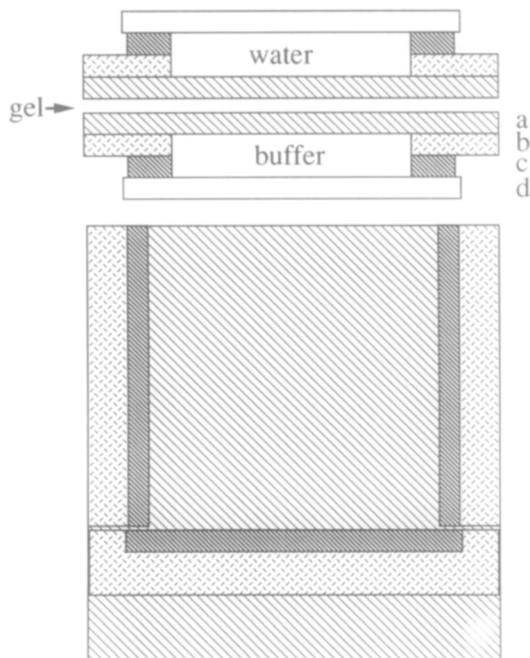


FIG. 3. Glass plate design for thermostatted DTE gels. A cross-section through the gel plate assembly is shown on the top. It consists of gel plates (a), wide spacers (b), narrow spacers (c), and buffer plate (d). Two sets of 4-mm thick glass spacers are used to give enough space for insertion of the thermostating tubing (see text). The wide spacers extend to the side of the gel plate to minimize temperature gradients; the second set of narrow spacers allows the use of normal clamps. A view from the top during glueing of the plate assembly is shown below; the buffer plate is omitted.

chamber dimensions setup is shown in Fig. 3, and the dimensions are given in Table I.

To glue spacers and buffer plate to the gel plate, the inner side of the gel plate is covered with a piece of bench coat to shield it, and spacers and buffer plate are glued to it with generous amounts of clear silicone rubber adhesive. The plate assemblies can be used after 2 days.

Casting of Gels for Direct Transfer Electrophoresis

General Comments. Several casting methods have been successfully used, including pouring from the top, sliding, and clapping. Especially for 0.1-mm thin long gels, the sideways clapping method described below works best for us.

TABLE I
DIMENSIONS OF GLASS PLATES, SPACERS, AND BUFFER PLATES FOR
THERMOSTATTED DIRECT TRANSFER ELECTROPHORESIS GELS

Apparatus	For 20 × 60 cm plate (cm)	Formula
Gel plates ^a	22 × 62.5	$w \times h$
Wide spacers		
Side	2.5 × 59	$2.5 \times (h - 3.5)$ cm
Bottom	2.5 × 22	$2.5 \times (w)$ cm
Narrow spacers		
Side	1.2 × 59	$1.2 \times (h - 3.5)$ cm
Bottom	1.2 × 19.5	$1.2 \times (w - 2.5)$ cm
Buffer plate	20 × 59.5	$(w - 2) \times (h - 3)$ cm

^a All glass plates are 4.7 mm (3/16 in.) thick; ears are 3–4 cm wide and 2.5 cm high.

Whatever method is used, it is essential to seal the lower surface from air to allow homogeneous polymerization; this is usually done with Parafilm and pressing of the gel assembly against a vertical surface. The second essential point is the careful alignment of glass plates at the bottom; badly aligned glass plates will give blurred band patterns.

While untreated glass plates can give good results, most consistent results were obtained when both glass plates were treated with bind silane. On reuse of bind silane-treated plates, sometimes significantly more blurry band patterns were observed; treatment of the glass plates with 0.5 M NaOH for 15–60 min after cleaning reverses these effects.

The usual precautions while handling acrylamide and bisacrylamide should be followed (wear gloves!).

Preparation of Glass Plates. Clean plates thoroughly with distilled water, ethanol, and Kimwipes. Lay plates horizontally and spread 0.5–1.0 ml of bind silane dilution onto each plate with Kimwipes. After 5–15 min at room temperature, wash off excess bind silane with ethanol and Kimwipes.

Remove any dust particles with flint-free paper tissue, slightly wet with ethanol.

Now attach the spacers to the glass plate without ears. When using clear, water-resistant adhesive tape (Tesa film 4204, Baiersdorf, Germany gives best results; Scotch tape 3750-G, 3M, St. Paul, MN, also works), press one or two strips onto the glass without introducing air bubbles between the tape and the glass. Especially for gels longer than 20 cm, the minimum width of the spacer should be 3 cm.

Alternatively, conventional plastic spacers can be used. Grease a 2-cm wide stripe on the sides of the glass plates without ears and one side

of the spacers; use a minimal amount of silicone vacuum grease and spread it evenly (wear gloves!). Put the spacer with the greased side down onto the glass plate and press it on over the whole length of the gel. The greasing prevents slippage of the spacers and reduces the introduction of air bubbles from the side when the sideways clapping technique is used.

Cover the glass plate–spacer assembly with the other (eared) glass plate. Align the plates and clamp them, using two clamps in the lower third. Insert the precomb (a flat piece of spacer material) about 3 mm deep into the top of the assembly, and fix it to the bottom plate with adhesive tape.

Now the bottom seal: turn the plate assembly around so that the eared plate is laying down. Fix a piece of Parafilm to the bottom of plate assembly with adhesive tape, leaving 2 cm at the sides (spacers) free. Bend the Parafilm around the lower edge of the gel, flip the plate assembly around, and lay it flat onto the casting rack or store it on a dust-free place for up to several days (a simple casting rack consists of a 3- to 5-cm high, 18 × 18 cm piece of Plexiglas with a second 18-cm wide piece of Plexiglas vertically screwed onto it¹¹; dimensions are for 22 × 22 cm glass plates).

Casting by Sideways Clapping. Prepare fresh acrylamide solution by dissolving acrylamide and bisacrylamide in urea buffer, and filter the solution. Before use, degas with stirring for 5–10 min; gels with at least 6% (w/v) acrylamide do not have to be degassed.

Check that you have the lower glass plate leveled, enough clamps (e.g., 15 for 60 × 20 cm gels), a rubber band to press the plate assembly against the vertical surface, and a stop watch.

Add TEMED (e.g., 40 μ l/50 ml) and 10% ammonium persulfate (e.g., 140 μ l/50 ml), mix, start the stop watch, and take 1 ml into an Eppendorf tube to control the polymerization time.

Lift the top glass plate (with ears) into a vertical position on the near side of the lower plate. Pour the acrylamide solution onto the lower plate next to the near spacer, starting in the middle of the plate and extending toward the top and the bottom. Slowly lower the upper glass plate, avoiding introduction of air bubbles.

Align the bottom of the plates carefully, checking the alignment with your fingertips. Set the first four clamps near the bottom of the gel. Check the alignment after each clamp and adjust it, if necessary. Now set the other clamps.

Press the gel assembly against the vertical piece of the casting rack and attach the rubber band.

Finally, set a weak clamp on the top of the gel. It should exert pressure onto the precomb; if this clamp is too strong, it may be difficult to insert the shark's-tooth comb later. For gels wider than 35 cm, we prefer a

different method to ensure a tight fit of the precomb. Using a 22-cm wide glass plate and a 3-kg weight, pressure is exerted over a wider region onto the notched glass plate directly on top of the precomb.

Check if the gel solution in the Eppendorf tube has polymerized yet. The casting should take 5–7 min, and the solution should start to polymerize in 12–18 min. If the polymerization times are significantly shorter or longer, adjust both TEMED and ammonium persulfate concentrations. When the polymerization starts while the gel is still being clamped, poor resolution will result due to a deformed gel matrix; it is probably better to discard these gels.

Let the gel polymerize for 1–24 hr (if the polymerization did not start within 15 min, let it sit for at least 2 hr). If the gel is stored overnight, squirt some water into the buffer chamber after the gel has polymerized, and cover the top with Saran wrap to prevent drying out of the upper gel surface; also recheck that the bottom is pressed tightly against a vertical surface.

Setup and Electrophoresis. Prewarm the electrophoresis buffer to 35–45°. Degas the buffer for the lower buffer chamber for at least 5 min with stirring in a vacuum sidearm flask. Clean the gel assembly and remove any excess acrylamide from the lower edges of the glass plates, but do not touch the lower surface of the gel.

Pour the degassed buffer into the lower buffer chamber and mount the gel in the electrophoresis unit; let it touch the buffer with one corner first to avoid trapping of air bubbles between gel and conveyor belt. Insert the thermostating tubing, and fill prewarmed buffer into the upper electrophoresis chamber and prewarmed water into the thermostating chamber, if thermostating is used.

Remove the precomb. Carefully remove all excess acrylamide from the top of the gel, using a syringe and, if necessary, a piece of comb material. Flush the upper gel surface with buffer.

The tips of the shark's-tooth comb may be greased lightly with silicone grease. Ungreased combs can give more problems with horizontal leakage, but too much grease interferes with sample loading and electrophoresis.

Insert the shark's-tooth comb; it should just touch the gel or go into it for about 0.5 mm. If there are problems inserting it, removing the uppermost clamps and supporting the comb with stiffer plastic may help. Practice loading or checking the wells with 50% stop buffer may be done now.

Preelectrophorese for 30–60 min at 70 V/cm for 0.1-mm gels and 60 V/cm for 0.2-mm gels. If you do not use thermostating, set an ampere (or watt) limit at about 25% more than the initial current (or wattage) to prevent thermal runaway.

TABLE II
TYPICAL RUNNING CONDITIONS AND RESOLVED BASES IN DIRECT
TRANSFER ELECTROPHORESIS

Length (cm)	Thickness (mm)	Gel concentration (%)	Voltage (V)	Membrane speed (cm/hr)	Resolved fragments ^a
20 ^b	0.2	6	1200	14	20–320
30 ^b	0.1	6	1800	9	20–420
30 ^c	0.1	4	2100	20	50–550
45 ^c	0.1	4	3150	13	50–600
60 ^c	0.1	3.5	4200	11	60–750

^a Typical results are given; optimal results can be better, and the interpretable range can also be larger. The acrylamide : bisacrylamide ratio is 19 : 1, except for 3.5% gels, for which a 34 : 1 ratio is used.

^b Gels run with one aluminum plate for temperature distribution; gel temperature is approximately 37°.

^c Gels run with thermostating from both sides at 50°.

Denature the samples immediately before loading for 2–3 min in an 80° waterbath, and quick-chill them on ice water. Flush the wells to remove urea, and load the samples with a duckbill pipette tip, a Hamilton syringe, or with a Hamilton 12-channel pipettor; the 12-channel pipettor is by far the easiest and most reliable of these. Start electrophoresis (60–70 V/cm; see above) immediately (if the loading takes longer than 5–10 min, start electrophoresis for 1–2 min between samples).

After the samples enter the gel and before the bromphenol blue reaches the gel bottom, shut off the power supply and move the nylon membrane under the gel. First mark the membrane by cutting an edge or writing on it with a suitable pen. Depending on the machine used, a dry membrane can be taped to the conveyor belt with waterproof tape, or a membrane wetted with distilled water is put underneath a strap on the conveyor belt.³⁴ Move the membrane below the gel and further under the first glass axle; this eliminates floating of the membrane in the buffer chamber.

Start the transport of the membrane shortly before the bromphenol blue reaches the membrane. Table II gives an overview of typical membrane speeds for several setups. Make sure that the membrane is transported out of the buffer at the end of the run.

Membrane Treatment after Electrophoresis. When radioactively labeled sequencing reactions are loaded, the membrane can be wrapped in thin plastic foil and exposed to X-ray film at room temperature; after the exposure, the nylon membrane can be stripped and reused as described.³⁴

For nonradioactive DNA sequencing and genomic or multiplex sequencing, put the membrane on clean Whatman (Clifton, NJ) 3MM paper

and dry it at 70–90° for 5–15 min. Covalently bind the DNA to the membrane by ultraviolet (UV) cross-linking.¹² Normal exposure times are 20 sec for multiplex and genomic sequencing and 30–120 sec for sequencing with end-label primers at 1.3 mW/cm² (UV boxes as described¹² and many UV transilluminators have light intensities of 0.6–1.5 mW/cm²). Store the membrane between clean Whatman 3MM paper sheets.

Especially when using nonradioactive deletion methods, always handle the membrane with clean gloves and avoid unnecessary mechanical stress and exposure to dirt; dust, fingerprints, bacterial contamination, forceps marks, and so on are likely to result in increased nonspecific background.

Apparatus Maintenance. After the run, take the gel out of the apparatus and replace the buffer in the lower buffer chamber with distilled water. Move the conveyor belt through the water four to six times to remove buffer residuals from the conveyor belt; change the water once between the washes.

Disassembly of Gel Sandwich. Insert a gel knife [#2117-161 (Pharmacia), the small blade of a Swiss army knife, or a razor blade] between the glass plates, about 2 cm below the ears. Insert slowly until the plates are far enough apart to allow the insertion of plastic wedges (#SE 1514; Hoefer, San Francisco, CA). Working with three or four plastic wedges from one side of the sandwich, pry the glass plates totally apart; take care that the bottoms of the plates are not damaged.

Use a piece of thin plastic (e.g., 0.35-mm thin polyester) to scrape the acrylamide from the glass plates; washing with warm water helps. Remove any remaining acrylamide with water and paper towels. Clean plates thoroughly with ethanol, water, and Kimwipes. Detergents are not necessary.

It may be necessary to immerse the glass plates in 0.5 M NaOH for 15–60 min to remove excess bind silane before reuse, followed by washing with water and ethanol.

Protocols for Nonradioactive DNA Sequencing

Oligonucleotide Labeling with Biotin and Digoxigenin

For biotin labeling of oligonucleotides, biotinylated phosphoramidites that can be used on an automatic synthesizer are most convenient. For labeling with digoxigenin and as a low-cost alternative to biotin phosphoramidites, oligonucleotides can be 5'-aminated during synthesis (e.g., with *N*-TFA-C₆-amino modifier; Clontech), followed by reaction with NHS-biotin or NHS-digoxigenin.

Both biotin and digoxigenin are hydrophobic, enabling purification by reversed-phase high-performance liquid chromatography (HPLC).^{35,36} Because both labels also reduce the electrophoretic mobility of oligonucleotides by an apparent size of 1–2 nucleotides, polyacrylamide gel electrophoresis can also be used for analysis of the reaction and for purification.³⁷ However, a purification is normally not necessary.

For maximum detection sensitivity, a sufficiently long spacer must separate the label from the oligonucleotide.^{27,36} With *N*-TFA- C_6 -amino modifier and NHS-XX-biotin (also called NHS-LC-biotin) or NHS-digoxigenin, this requirement is fulfilled.

Labeling of Aminated Oligonucleotides with Biotin. Dissolve the deprotected and lyophilized oligonucleotide in water (180 μ l for 0.2- μ mol synthesis scale, 900 μ l for 1.0- μ mol synthesis). Mix 90 μ l (0.1 μ mol) with 10 μ l 1 *M* sodium carbonate, pH 9.0, and add 100 μ l of biotin-XX-NHS ester (20 mg/ml in dimethylformamide, freshly prepared). Incubate at 42° for 2 hr; the formation of a precipitate is normal and can be ignored. Add 300 μ l 0.1 *M* Tris-HCl, pH 8.0, and incubate at room temperature for 1 hr.

Remove excess biotin by gel filtration through Sephadex G-25 (NAP-5 columns; Pharmacia) with 0.1 *M* triethylamine acetate, pH 7.0, as buffer, lyophilize the eluate, and resuspend in TE. The oligonucleotide can be analyzed by gel electrophoresis as described below; we usually obtain biotinylation efficiencies of 50–90%. The oligonucleotides can be used directly or purified by HPLC or gel electrophoresis.

Labeling of Aminated Oligonucleotides with Digoxigenin. The labeling is done essentially as described for the biotinylation, except that 200 μ l of NHS-digoxigenin (6.25 mg/ml, freshly dissolved in dimethylformamide) is added to 90 μ l of oligonucleotide (0.1 μ mol) and 10 μ l of 1 *M* sodium carbonate, pH 9.0; again, a precipitate may form and can be ignored. After reaction overnight at room temperature, add 250 μ l of 0.2 *M* Tris-HCl, pH 8.0, incubate for 1 hr at room temperature, and purify through NAP-5 columns as above. The reaction yield is about 50%.

Analysis of Labeling Reaction by Gel Electrophoresis and Staining with "Stains-All." Mix 0.5–1 nmol of labeled oligonucleotide with an equal volume of deionized formamide containing 0.04% bromphenol blue, and load onto a 15% denaturing acrylamide gel (e.g., 20 cm \times 20 cm \times 0.8 mm). Electrophorese at 40 V/cm until the bromphenol blue reaches the lower quarter of the gel (about 1 hr). Disassemble the glass plates, and

³⁵ A. J. Cocuzza, *Tetrahedron Lett.* **30**, 6287 (1989).

³⁶ K. Mühlegger, E. Huber, H. von der Eltz, R. Rürger, and C. Kessler, *Biol. Chem. Hoppe-Seyler* **371**, 953 (1990).

³⁷ A. Chollet and E. H. Kawashima, *Nucleic Acids Res.* **13**, 1529 (1985).

stain the gel in $1\times$ Stains-all until bands are clearly visible (about 5–20 min). Destain the gel in distilled water for 15–60 min; prolonged exposure to daylight reduces signal intensities. Document the results by photography, or by photocopying the gel sandwiched between clear plastic sheets.

Sequencing Reactions

The sequencing reactions can be done with a one-step protocol because no labeling reaction is necessary. Best results are obtained with single-stranded template DNA from recombinant phagemid clones (in pTZ18R; Pharmacia) or M13, using modified T7 DNA polymerase³⁸ and manganese isocitrate buffers.³⁹ The uniform band intensities with this system simplify interpretation of the sequence patterns and often allow reliable reading even in regions where multiple bands are badly resolved. Double-stranded template DNA, and different DNA polymerases and protocols can also be used.^{11,14}

The ratio of deoxynucleotides to dideoxynucleotides must be adjusted; with manganese isocitrate buffers and T7 DNA polymerase, a ratio of 250 : 1 gives good sequence patterns up to more than 1000 bases. If the main interest is the first 300 bases, a ratio of 125 : 1 can be used.

Primer Annealing

1. Combine 0.5 pmol of single-stranded template DNA, 0.5 pmol of end-labeled primer and 2 μ l of annealing buffer in a total volume of 13 μ l.
2. Place in a 65–75° water bath for 2–3 min.
3. Cool slowly to room temperature over 15–45 min; use directly or store at –20°.

Sequencing Reactions

1. Add 1 μ l of 0.1 M DTT to the annealing mixture and mix carefully; the color changes to dark brown and then to clear.
2. Distribute 2.5 μ l of the nucleotide mixes into reaction vials (Eppendorf tubes, Terazaki plates, polypropylene plates, or polystyrene microtiter plates) and prewarm in a 37–40° water bath for 1–2 min.
3. Dilute modified T7 DNA polymerase to 1.5 U/ μ l with ice-cold TE.
4. Add 2 μ l of enzyme to the annealing mixture, mix carefully, and distribute 3.5- μ l aliquots to the prewarmed nucleotide mixes; incubate at 37–40° for 2–15 min.
5. Stop the reactions by adding 6 μ l formamide–dye mix; the reactions can be stored at –20° for several months and loaded repeatedly.

³⁸ S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4767 (1987).

³⁹ S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4076 (1989).

6. Immediately before loading, denature the sequencing reactions for 2 min in an 80° waterbath, chill on ice-water, and load directly.

Nonradioactive Detection of DNA Sequencing Patterns

General Comments. All buffers used should be made with the highest purity water available and kept free of contamination with external enzymes; fingerprints and bacteria, for example, are good sources of alkaline phosphatase. Most of the buffers used, including SDS buffer, support growth of bacteria. Addition of 1 mM EDTA to Triton and SDS buffers shows sufficient bacteriostatic effects, but it can also lead to twofold reduced signal; the most foolproof way is to prepare all 1× buffers immediately before use. Additional background problems can arise from lint and dirt and from mechanical damage of membranes. Membranes should be handled carefully with clean gloves and kept between Whatman 3MM paper (or in hybridization bags) before the detection.

The detection protocols can be carried out in hybridization bags, large plastic trays, or large cylinders.^{11,40} All methods avoid overlapping of membrane parts that can lead to reduced signal and increased background in nonradioactive protocols.

Development in hybridization bags with a small “chimney” for exchange of solutions offers the advantage that chemiluminescent exposures can be done by putting an X-ray film on top of the bag; however, the thickness of the bags leads to more blurry sequence patterns. Further problems can arise from uneven distribution of enzyme or substrate solutions and trapped air bubbles.

Large, flat plastic trays on gyratory shakers are convenient for the development of small numbers of membranes at a time. They can give the most efficient washing from both sides of the membrane; however, somewhat higher volumes of all solutions are needed.

Large cylinders on horizontal rollers are ideal to minimize the volume of valuable incubation solutions. Simultaneous development of several membranes is easy, and automation of the development process is straightforward.¹¹ A variety of simple home-made rollers, cell culture rollers, or hybridization ovens can be used for this purpose. A more sophisticated machine that uses up to four polycarbonate bottles with magnetic bottoms and is programmable for tilt angle, revolution speed, and step time for up to 484 steps (Western Roller-4; BioComp) is routinely used in our laboratory.

⁴⁰ N. Thomas, C. N. Jones, and P. L. Thomas, *Anal. Biochem.* **170**, 393 (1988).

For All Detection Protocols. Unless otherwise indicated, all washing steps are done for 2–5 (up to 20) min with 250–350 ml of buffer (approximately 500 ml in trays) for 32 × 40 cm membranes. The numbers of washing steps given below are minimum numbers; if background is a problem, up to four washing steps at each point and careful removal of solutions between steps can be helpful.

Developments in cylinders are done at 5–10 rpm. When trays are used, the shaker speed is adjusted so that the membrane is completely covered with solution (25 to 75 rpm); in incubations with streptavidin, enzymes, and substrates, it is advantageous to distribute the solutions by manual shaking every 2 to 3 min.

1. After drying and UV cross-linking, fit the membrane in a plastic or siliconized glass cylinder with the DNA facing inward.

2. Rehydrate the membrane with distilled water; try to avoid or remove air bubbles between the membrane and the cylinder.

Protocol 1: Detection of Biotin-Labeled Sequence Patterns

3. Incubate with SDS buffer for 5–30 min.

4. Incubate with streptavidin in SDS buffer (1 $\mu\text{g}/\text{ml}$, 50 ml) for 10 min.

5. Wash two times with SDS buffer.

6. Wash once with Triton buffer.

7. Incubate with biotinylated alkaline phosphatase in Triton buffer (1 $\mu\text{g}/\text{ml}$, 50 ml) for 10 min.

8. Wash two times with Triton buffer.

Protocol 1a: Chemiluminescent Detection

9. Wash two times with 1× DEA buffer.

10. Incubate with 50 ml dioxetane substrate solution for 5 min.

11. Discard the substrate solution, take the membrane out of the cylinder, and let it drip dry for 30–60 sec. Wrap it in clear plastic foil (Saran wrap), avoiding wrinkles and air bubbles between membrane and foil.

12. Expose to X-ray film; exposure times depend on the amount of DNA loaded; 5–30 min is typical. Please note that the signal per time unit increases for a couple of hours with dioxetane substrates³³; a 15-min exposure after 60 min will give about four times more intense bands than an immediate 15-min exposure.

Protocol 1b: Colorimetric Detection

This protocol can also be used after chemiluminescent detection; two additional washes with Triton buffer can then be added before step 9.

9. Wash the membrane twice with $1 \times$ Tris-HCl, pH 9.5.
10. Incubate with BCIP/NBT solution until bands are sufficiently dark (20 min to overnight).
11. Wash the membrane twice with Triton buffer for at least 15 min (up to overnight).
12. Wash the membrane with distilled water.
13. Dry the membrane on Whatman 3MM paper at $70-90^\circ$ for 5–15 min. Store the membrane in a hybridization bag in a dark place (e.g., between black cartons).

Protocol 2: Detection of Digoxigenin-Labeled Sequence Patterns with Phosphatase–Antibody Conjugates

Start with step 3.

3. Incubate the membrane with 100 ml block buffer for 30–60 min (shorter times may be sufficient).
4. Incubate with 50 ml of anti-digoxigenin–alkaline phosphatase conjugates (150 mU/ml) in block buffer for 30 min.
5. Wash twice with block buffer.
6. Proceed with colorimetric or chemiluminescent development as described in protocols 1a and 1b, respectively.

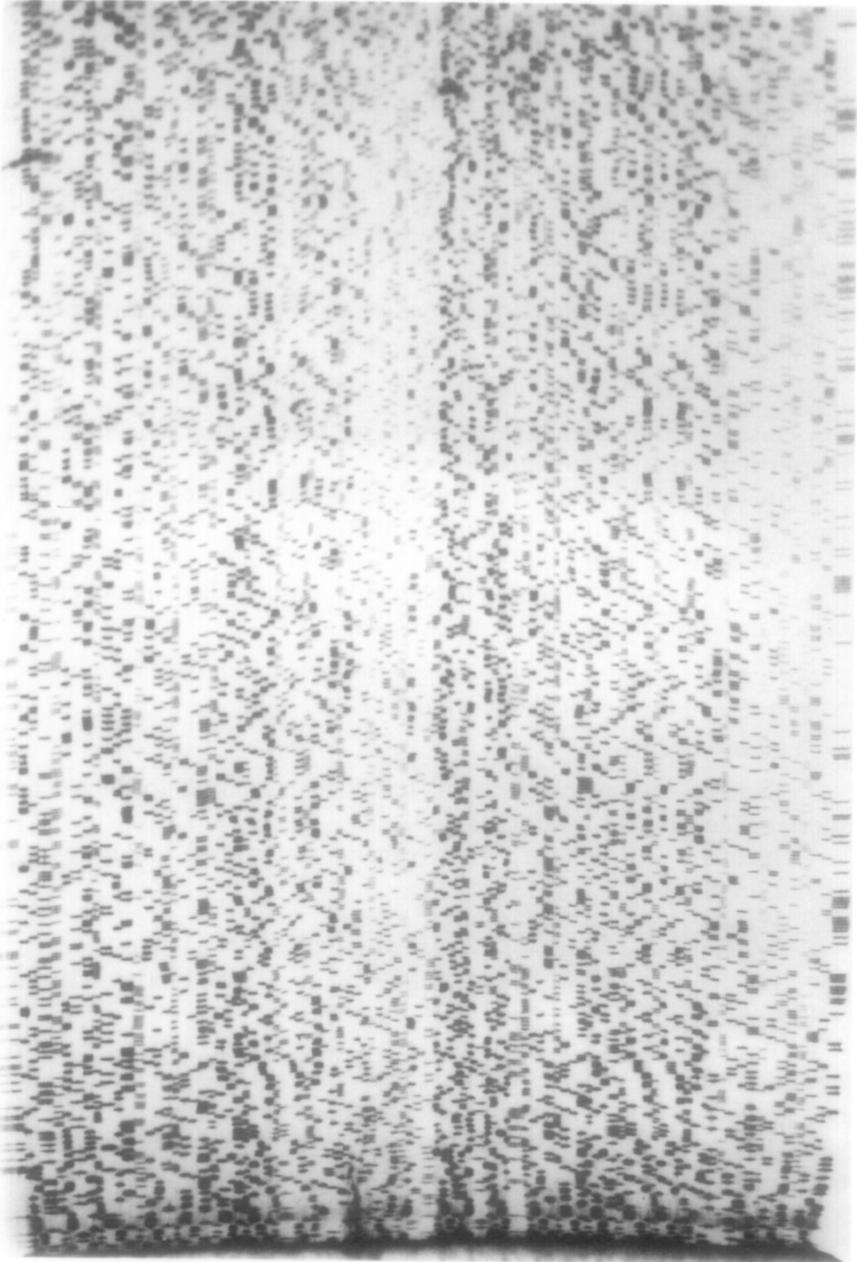
Protocol 3: Detection of Digoxigenin-Labeled Sequence Patterns with Peroxidase–Antibody Conjugates

Start with step 3.

3. Incubate the membrane with 100 ml block buffer for 30–60 min (shorter times may be sufficient).
4. Incubate with 50 ml of anti-digoxigenin–peroxidase conjugates (150 mU/ml) in block buffer for 30 min.
5. Wash twice with block buffer.
6. Wash twice with 0.1 M Tris-HCl (pH 8.0), 150 mM NaCl.
7. Decant, add 50 ml ECL detection solution 2, and mix; add 50 ml ECL detection solution 1, and mix for 1 min.
8. Wrap membrane in clear plastic foil (Saran wrap) and expose to X-ray film. An initial exposure of 1 min can give an indication of the exposure time needed (usually 10–45 min). Please note that the signal intensity of peroxidase-based enhanced chemiluminescence is maximal in the first minutes after substrate addition and then decays over 1–2 hr.³⁰

Protocol 4: Combined Protocols for Successive Detection of Biotin- and Digoxigenin-Labeled Sequence Patterns

Before denaturing and loading onto a polyacrylamide gel, combine equal amounts of two sequencing reactions, one with a biotinylated primer



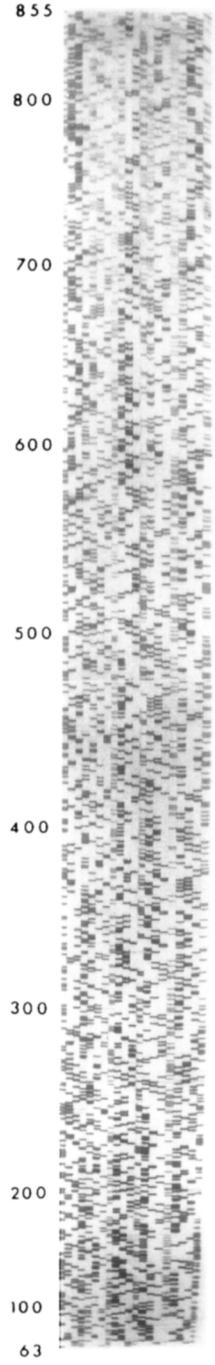
and one with a digoxigenin-labeled primer. Alternatively, differently labeled "universal" and "reverse" primer can be used for sequencing of double-stranded templates. Dry, cross-link, and rehydrate the membrane as described above. Start with step 3.

3. Incubate with SDS buffer for 5–30 min.
4. Incubate with streptavidin in SDS buffer (1 $\mu\text{g}/\text{ml}$, 50 ml) for 10 min.
5. Wash two times with SDS buffer.
6. Wash once with Triton buffer.
7. Incubate with biotinylated alkaline phosphatase (0.5 $\mu\text{g}/\text{ml}$, 50 ml) in block buffer for 20 min.
8. Incubate with anti-digoxigenin–peroxidase conjugates (150 mU/ml, 50 ml) in block buffer for 30 min.
9. Wash two times with block buffer.
10. Wash twice with 0.1 M Tris-HCl (pH 8.0), 150 mM NaCl.
11. Decant, add 50 ml ECL detection solution 2, and mix; add 50 ml ECL detection solution 1 and mix for 1 min.
12. Wrap membrane in clear plastic foil (Saran wrap) and expose to X-ray film (15–45 min).
13. Wash the membrane two times with Triton buffer.
14. Wash twice with 1 \times DEA buffer.
15. Incubate with 50 ml dioxetane substrate solution for 5 min.
16. Wrap membrane in clear plastic foil and expose to X-ray film.

Protocol 5: Membrane "Stripping" after Phosphatase-Based Detections

To denature (or remove) alkaline phosphatase from a previous development, the membrane is treated two times with stripping buffer, preheated to 65–75°, for 15 min. Most conveniently, this step is done either in a sealed hybridization bag in a water bath or hybridization oven, or in a tray that has been prewarmed with hot distilled water and covered with a plastic sheet to reduce temperature drops due to evaporation. The effectiveness of the stripping procedure can be checked by washing the membrane with 1 \times DEA buffer, incubation with dioxetane substrate, and exposure to X-ray film.

FIG. 4. Chemiluminescence-detected sequencing patterns from direct transfer electrophoresis with 45 \times 45 cm gels. Twenty-four sequencing reactions with biotinylated primers were loaded onto a 4% acrylamide gel and detected as described in protocol 1a, using a plastic tray. The original size of the membrane was 32 \times 40 cm.



Results

Figures 4 and 5 show sequencing patterns obtained with direct transfer electrophoresis and chemiluminescent detection. In Fig. 4, 24 reactions were loaded onto a 45×45 cm 4% acrylamide gel; about 400 nucleotides per lane set can be read, giving almost 10,000 bases of sequence information from one film. The pattern shown in Fig. 5 was generated with a 60-cm long, 20-cm wide 3.5% acrylamide gel. A constant interband spacing for fragments between 200 and 850 nucleotides in length was obtained with a constant membrane speed. For shorter fragments, the bands are more closely spaced; because these bands also tend to be sharper, the sequence can be read accurately. With increasing fragment length, the bands become broader until multiple bands are poorly resolved; an enlarged section from the top of Fig. 5 is shown in Fig. 6 to illustrate this point. Due to the constant and sufficient spacing between bands and the even band intensities obtained with modified T7 polymerase and manganese buffers,³⁹ the correct sequence can be obtained even in this top region, giving about 790 nucleotides of sequence information per lane set. For different gel parameters, exemplary numbers of resolved bases are given in Table II.

Figures 4 and 5 also show that virtually background-free sequence patterns can be obtained with chemiluminescent detection. For the data shown in Fig. 5, three sequencing reactions with differently labeled primers and 1 pmol of template DNA per reaction were combined, giving a total volume of $36 \mu\text{l}$; about $1 \mu\text{l}$, that is $1000 \text{ fmol}/(4 \text{ lanes} \times 36 \mu\text{l} \text{ total}) = 7 \text{ fmol}$ was loaded per lane. With a 250:1 ratio of deoxy- to dideoxynucleotides and no discrimination between deoxy- and dideoxynucleotides,³⁹ the amount of DNA in the first bands is about 0.03 fmol (20 million molecules).

The patterns shown in Figs. 5 and 6 were the second patterns produced from this membrane; the membrane has been developed with the combined protocol for the successive detection of digoxigenin- and biotin-labeled

FIG. 5. Chemiluminescence-detected sequencing patterns from a 60×20 cm, 3.5% acrylamide gel. The length of the original membrane was 82 cm. Five lane sets are shown; the fragment length is indicated on the left. Three different sequencing reactions with biotin-, fluorescein-, and digoxigenin-labeled primers were mixed before loading. After electrophoresis at 4200 V with a constant membrane speed of 11 cm/hr, the sequencing patterns were detected as described in protocol 4, except that 1% casein in $1 \times$ Triton buffer was used instead of block buffer ("essentially vitamin-free" casein from Sigma was dissolved in 0.1 M NaOH and neutralized by addition of 100 ml 1 M Trizma-HCl before addition of $10 \times$ Triton buffer and filtration). The pattern shown is from the streptavidin-biotinylated alkaline phosphatase detection with AMPPD; the exposure time was 20 min, starting 33 min after substrate addition. Enlarged sections of this film are shown in Fig. 6 (lanes 5-8 from the left, nucleotides 735-855) and Fig. 7b (nucleotides 336-417).

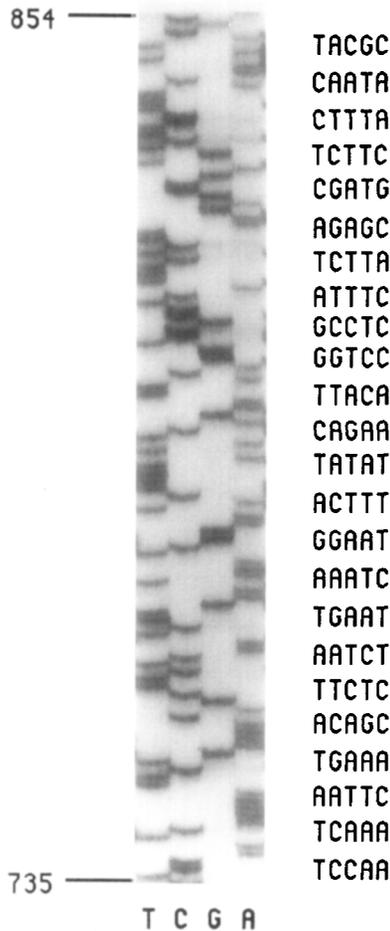
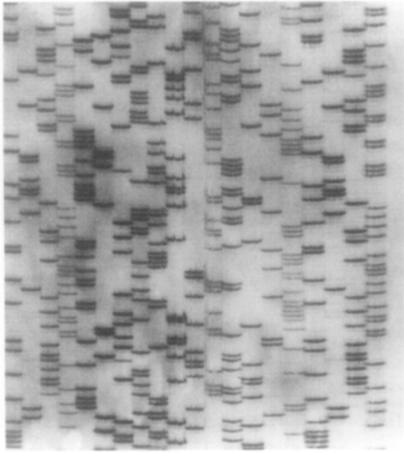


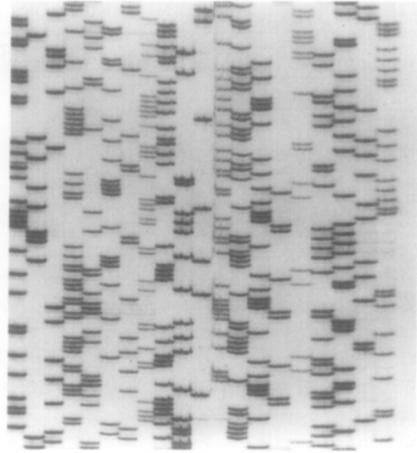
FIG. 6. An enlarged section from the top of Fig. 5, showing the region where poor band separation begins to limit reliable reading. The total length of the fragments is indicated on the left, the correct sequence on the right.

primers (protocol 4). Figures 7a and b show the results of these detections for a section of the membrane; two independent sequencing patterns can be obtained within less than 4 hr after electrophoresis.

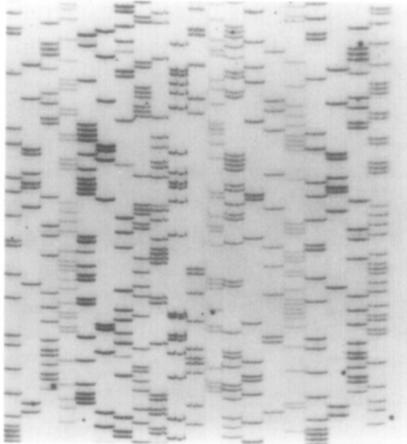
After these first two detections, the membrane has been "stripped" to remove phosphatase activity and anti-digoxigenin-peroxidase conjugates from the membrane (protocol 5) and then developed with anti-digoxigenin-phosphatase conjugates and AMPPD-Cl (protocol 2). The results in Fig. 7c show that the phosphatase activity from the biotin-based detec-



**a. anti-DIG-HRP
ECL**



**b. streptavidin
biotin-CIAP
AMPPD**



**c. anti-DIG-CIAP
AMPPD-Cl**

FIG. 7. Sequential detection of DNA sequencing patterns with digoxigenin-labeled primer, anti-digoxigenin antibody-peroxidase conjugates and enhanced chemiluminescence (a), biotin-labeled primer, streptavidin-biotinylated alkaline phosphatase and AMPPD (b), and anti-digoxigenin antibody-alkaline phosphatase conjugates and AMPPD-Cl (c). Exposure times were (a) 45 min, (b) 20 min (starting 33 min after substrate addition), and (c) 30 min (starting 30 min after substrate addition). The detection was done with the combined protocol (protocol 4 with modifications as noted in the caption to Fig. 5) for (a) and (b), followed by stripping as described in protocol 5 and detection as described in protocol 2. The total length of the DNA fragments in the section shown ranges from 336 nucleotides at the bottom to 417 nucleotides at the top.

tion can be removed well enough to allow two subsequent phosphatase-based detections.

Discussion

Comparison of Direct Transfer Electrophoresis and Other Electrophoresis Methods for DNA Sequencing

In DNA sequencing, the main priorities can vary significantly. When teaching or learning the technique or when sequencing is done only occasionally, simple and reliable systems can be a priority. For high-throughput projects, highly optimized systems that emphasize long reads for the resulting higher overall efficiency are useful. Under both circumstances, nonradioactive detection methods may be preferred to radioactive labeling because of reduced health hazards, fewer regulatory problems, more stable reagents and reactions, and faster detection.

Direct transfer electrophoresis offers considerable advantages for all these needs. For learning and occasional sequencing, very short gels that are easy to cast and handle can be useful; 20-cm long gels can resolve fragments up to 400 nucleotides in length, comparable to 40- to 55-cm long gels in conventional electrophoresis. When higher performance is desired, one 60-cm long gel can resolve fragments up to 800 nucleotides in length; to obtain similar results with conventional electrophoresis, multiple loadings on two or three 80- to 100-cm long gels are necessary.⁵

The membrane-bound sequencing patterns generated by direct transfer electrophoresis are also an ideal starting point for enzyme-linked detection methods. Colorimetric detections are cost competitive, and chemiluminescent detection can give results within less than 2 hr after electrophoresis. If high efficiency is of importance, the combination of two different label-detection systems as shown offers a simple way to double the information per sequencing gel, independent of other parameters like slot width or resolved bases; for highest demands, direct transfer electrophoresis can be combined with multiplex sequencing.¹³

Another step toward higher sequencing efficiency can be the elimination of the manual sequence reading; this can be achieved by using film scanners²³ or fluorescence-based machines. Because machines for direct transfer electrophoresis are technically much simpler than those for fluorescent sequencing, several DTE machines and a film scanner can be obtained for the price of one fluorescence-based automate. Because most film scanners can analyze at least 10 films/day,²³ the DTE-plus-scanner setup could give a significantly higher performance.

Practical Aspects: Gel Length, Gel Thickness, and Thermostatting

Direct transfer electrophoresis is highly flexible; to learn the method, short, convenient gels can be used, possibly in combination with standard radioactive sequencing. Later, the system can be easily changed and expanded according to the preferences of the experimenter, for example, to nonradioactive duplex sequencing with "high performance" 60-cm gels.

A critical point in DTE is the lower gel surface; it may take a few trials to produce reproducibly good gels. Our success rate on casting 0.1-cm thin, 20 × 60 cm or 45 × 45 cm gels is above 90%.

For learning and some routine applications, 0.2-mm thick gels can be more convenient than 0.1-mm gels, for which sample loading can be somewhat tedious. The use of 12-fold Hamilton syringes simplifies loading considerably, especially on 0.1-mm gels; 96 lanes can be loaded in less than 10 min. If longer reads or sharper band patterns are desired, we recommend using 0.1-mm thin gels.

Thermostatting of direct transfer electrophoresis gels is not mandatory. Good results can be achieved by using an aluminum plate or a buffer chamber that extends toward the bottom of the gel for heat distribution. However, we prefer to use thermostatted gels because of several advantages.

1. Gels can be run at higher temperatures without increased problems with "thermal runaway" (with voltage regulation, the power generated heats the gel, the electrical conductance increases, higher power heats the gel even more . . . until the buffer boils or glass plates crack). The normal countermeasure against thermal runaways is to use power instead of voltage regulation; however, this often leads to reduced voltages during the run, resulting in increasing interband distances in DTE. Higher temperature reduces the number of compression artifacts and also leads to faster runs; the mobility of DNA increases by about 2%/°C.⁴¹

2. Gel temperature and voltage can be regulated independently. This helps to obtain reproducible results when experiments are run at different room temperatures, and it is especially useful for systematic studies.

3. Thermostatting from both sides reduces the temperature gradient that can form over the width of the gel; such temperature gradients can lead to broader band patterns.⁴¹

The thermostatting method as described here is simple to implement, electrically insulated and efficient. A simple, noncooling, pumping water bath is sufficient and can be used for two DTE machines simultaneously.

⁴¹ R. J. Wieme, in "Chromatography" (E. Heftman, ed.), 2nd Ed., p. 210. Reinhold, New York, 1967.

Theoretical Aspects of Gel Parameters in Direct Transfer Electrophoresis

The following discussion is simplified, intended to answer some common questions about direct transfer electrophoresis; more explicit discussions of some principles can be found elsewhere.⁴¹⁻⁴⁵

What determines the range of constant interband spacing in direct transfer electrophoresis? The linear range in DTE is mainly determined by the gel concentration and the electric field. Lowering the acrylamide concentration increases the linear range toward longer fragments, whereas increased field strength reduces it (see below). Because lower acrylamide concentrations also reduce the resolution between fragments, an optimal concentration for practical purposes can be found; with 60-cm long gels, 3.5% acrylamide gives best results, whereas 3.0% gels are slightly inferior. For shorter gels, higher concentrations can be optimal. Similar optimal gel parameters were found in capillary electrophoresis.⁴⁶

Does the transfer process itself lead to the blurry bands? Why do thinner gels give better results. With carefully polished and aligned glass plates and DTE machines that give good contact between lower the gel surface and the membrane, the broadening of bands during the transfer process due to diffusion is negligible. This has been shown with machines that move the membrane in steps slightly larger than the gel thickness,¹¹ resulting in "striped" bands due to membrane movements during the transfer of one band. Typical step sizes in our DTE machines are around 30 μm .

However, bands on the membrane cannot be thinner than the thickness of the gel. With 0.35-mm thick gels and normal membrane speeds, the data quality is significantly reduced; 0.2-mm gels give much sharper bands, and a slight further enhancement of data quality can be found with 0.1-mm thin gels.

Thinner gels also reduce the temperature gradient that can form over the width of the gel, thereby further improving the data quality.

How much (and how) can longer gels improve the readable range? With high-quality sequencing reactions and electrophoresis systems such as DTE, the length of the readable patterns is not restricted by the gel size

⁴² J. C. Giddings, *Sep. Sci.* **4**, 181 (1969).

⁴³ J. J. Kirkland, W. W. Yau, H. J. Stoklosa, and C. H. Dilks, Jr., *J. Chromatogr. Sci.* **15**, 303 (1977).

⁴⁴ J. W. Jorgenson and K. D. Lukacs, *Anal. Chem.* **53**, 1298 (1981).

⁴⁵ H. Swerdlow and R. Gesteland, *Nucleic Acids Res.* **18**, 1415 (1990).

⁴⁶ J. A. Luckey, H. Drossman, A. J. Kostichka, D. A. Mead, J. D'Cunha, T. B. Norris, and L. M. Smith, *Nucleic Acids Res.* **18**, 4417 (1990).

itself; instead, the reliable interpretation of sequencing pattern becomes tedious or impossible because of badly overlapping, unresolved bands. Important factors that contribute to the broadness of bands are (1) random distortions, for example diffusion, (2) loading of samples, (3) the transfer process, (4) the detection process, and (5) possibly other factors.

To examine the expected dependence of the gel length on the single factors contributing, we do a *Gedankenexperiment* (thought experiment) with direct transfer electrophoresis, keeping all conditions such as field strength, and so on, constant, but varying the gel length and the membrane speed; the membrane speed is adjusted so that the same distance between bands is achieved regardless of the gel length.

The elution time t_e for fragments is proportional to the gel length g and inversely proportional to the electrophoretic mobility m ; in the linear range, t_e can be described as

$$t_e = g(k_1 n + k_2) = g/m \quad (1)$$

Random distortion: Diffusion (and similar random effects during electrophoresis) induce variance according to Einstein's law, $\sigma_{\text{dif}}^2 = 2Dt$; D is the effective diffusion coefficient under electrophoresis conditions.⁴² Starting with a band of negligible width, a gaussian peak with width

$$4\sigma_{\text{dif}} = (32Dt)^{1/2} \quad (2)$$

will result at time t . Increasing the gel length g increases the elution time proportionally; also, the distance and elution time difference between two adjacent bands increases in proportion to g .

The resolution between two bands is defined as the ratio of their distance to their average width⁴²; an equivalent definition can be made on the time scale:

$$R = gk_1/t_w \quad (3)$$

where gk_1 is the elution time difference between two bands and t_w is the "time width," $4\lambda/m$. Thus,

$$R = mgk_1/4\sigma \quad (4)$$

or, with Eq. (2),

$$R = mgk_1(32Dt_3)^{-1/2} \quad (5)$$

We define $k_3 = mk_1(32D/m)^{-1/2}$; because $t_e = g/m$, this gives

$$R = k_3 g^{1/2} \quad (6)$$

This gives a square root relation between resolution and gel length at a given fragment length n . Increasing the gel length fourfold would result in

twofold better resolution at a given point; for gaussian bands of equal intensity, previously unresolved peaks (with $R = 0.5$) would be clearly resolved ($R = 1.0$, the intensity between two peaks would drop to 27% of the maximum).

Loading effects: samples are loaded at a given height, usually 1–3 mm; while entering the gel, this zone is compressed, but of course does not become one-dimensional. This compression is independent of the gel length; however, the membrane speed in our *Gedankenexperiment* is indirectly proportional to the gel length. If loading effects were the only broadening effects, a fourfold longer gel with fourfold lower membrane speed would compress the band width to 25%. The loading effects are inversely proportional to the gel length.

Transfer process: As discussed before, broadening due to the transfer can be easily minimized by using thin, well-aligned gels. The theoretical treatment is just as simple: transfer broadening is independent of the gel length. Its effects on the resolution are, however, dependent on the membrane speed under given conditions and gel length (a variation of the above *Gedankenexperiment*): faster transport would increase the distance between bands, simultaneously increasing the resolution.

Detection process: The detection process can contribute to the band broadening by giving wider bands; think, for example, of overexposures with ^{32}P or chemiluminescent substrates. The dependences on gel length and membrane speed are the same as for transfer broadening: independent of the gel length, but increasing interband distances by higher membrane speeds would reduce these effects.

Combined effects of different factors: The variance introduced by different mechanisms such as diffusion (σ_{dif}^2), loading (σ_{id}^2), transfer (σ_{tr}^2), detection (σ_{det}^2), and other possible sources (σ_{oth}^2) is additive:⁴³

$$\sigma_{\text{tot}}^2 = \sigma_{\text{dif}}^2 + \sigma_{\text{id}}^2 + \sigma_{\text{tr}}^2 + \sigma_{\text{det}}^2 + \sigma_{\text{oth}}^2 \quad (7)$$

The width of a band, assuming gaussian shape, is four times the square root of the variance, $4\sigma_{\text{tot}}$. If we assume that random distortions such as diffusion contribute mainly to the broadness of bands, we would expect a square root dependency of the resolution on the gel length; this would obviously give sharper band patterns for all fragment lengths and thus increase the readable range. Another way to reduce diffusion effects would be to use higher field strengths to reduce elution time; again, a square root relation between resolution and field strength would be expected. However, Fig. 8 shows that increasing field strengths also lead to reduced distances between bands at long fragments, as is well known for agarose

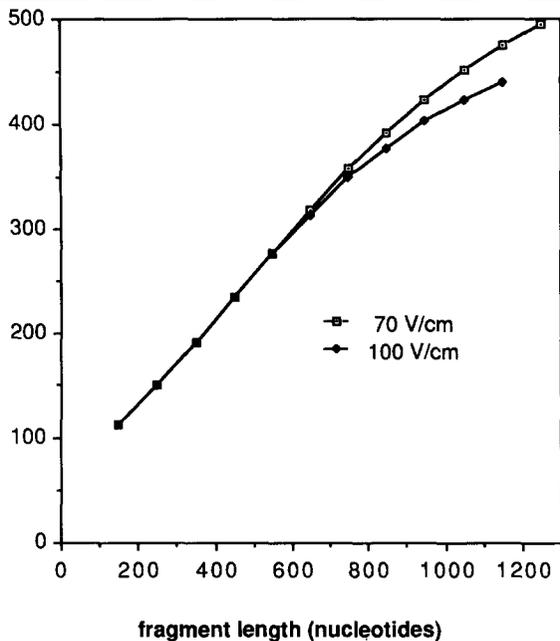


FIG. 8. Elution time as a function of fragment length in direct transfer electrophoresis at 70 V/cm (\square) and 100 V/cm (\blacklozenge); 0.1-mm thin, 60-cm long 3% acrylamide gels (3% total/5% bisacrylamide) were used. To allow a comparison of the linearity of the elution times, the times at 100 V/cm were normalized to the 70 V/cm times by multiplication with 1.551.

gels.^{47,48} This can practically limit the beneficial effects of higher field strength and may (at least partly) explain why capillary electrophoresis can give sharp bands for short fragments, but badly overlapping bands at longer fragments.⁴⁵

Another implication of the curvature for long fragments in Fig. 8 is that it becomes increasingly difficult to obtain well-separated bands with increasing fragment length. However, the optimal gel parameters given in this chapter do not represent a physical limit; further improvements are likely.

How is the transfer speed determined? Can the readable range be increased by spreading the bands with higher membrane speeds? Higher membrane speeds increase the distances between bands, but they also spread the bands out farther; the resolution as defined by the distance

⁴⁷ E. M. Southern. *Anal. Biochem.* **100**, 319 (1979).

⁴⁸ N. C. Stellwagen. *Biochemistry* **22**, 6180 (1983).

between bands divided through their width is not improved—if the interband distances are large enough to make broadening due to the transfer and the detection process negligible. Increasing the distances between bands also increases exposure times and reduces the data density, both unwelcome effects. As a compromise, the transfer speed is usually chosen to give interband distances between 0.8 and 1.4 mm. Thin gels (0.1 mm) and colorimetric detection (or ^{35}S) allow distances closer to 0.8 mm, whereas thicker gels and chemiluminescent detection (or ^{32}P) can benefit from slightly larger distances.

Why are bands from short fragments straight, but become more and more distorted with increasing fragment length? The most typical reasons for band shape distortions are small irregularities in the upper (and sometimes lower) surface of the gel; when, for example, shark's-tooth combs are used, the slots and bands often show a U shape. Such shapes are imprinted into the band shape. Within the gel, they have the same form for all bands regardless of fragment size. During transfer at the bottom of the gel, these distortions become enlarged according to the speed of the membrane in relation to the speed of a band; with constant membrane speed, the distortions become increasingly enlarged with decreasing mobility for longer fragments.

Increasing the gel length and keeping interband distances constant allows reduction of the membrane speed; therefore, images of distortions on the gel edges are reduced, and the sequencing patterns from longer gels are easier to interpret, especially for longer fragments.

Enzyme-Linked Detection Methods in DNA Sequencing:

Common Questions

How much DNA is needed for enzymatically detected DNA sequencing? We routinely use standard amounts, 0.5 pmol of single-stranded template DNA, per reaction; less than one-tenth of a reaction is loaded on 0.1-mm thin gels, and the nucleotide mixes are optimized for long reads—the amount per band in typical radioactive or fluorescent sequencing gels is often severalfold higher. An indication of the possible sensitivity of the methods was obtained when we determined the biotinylation rate of a primer that gave a weak, but clearly readable, sequence: only about 1.2% of the primer was biotinylated; the amount of biotinylated DNA per band was calculated to be below 0.001 fmol (0.6 million molecules).

How reproducible, efficient, and cost effective is nonradioactive sequencing? Following the protocols and general guidelines given in this chapter, we routinely obtain easily interpretable sequencing patterns,

often completely free of background; however, we usually use slightly more washes than given as minimum numbers in the protocol. Most problems, for example, in undergraduate courses, could be explained by use of old, bacterially contaminated buffers or inadequate membrane treatment.

When done routinely, the development process takes about 1 hr before the exposure or the start of the color reaction; efficiency can be easily increased by developing more membranes in parallel; four membranes can be developed in about 90 min. The combined detection protocol for differently labeled primers offers even more efficiency by reducing the time per protocol and the number of gels.

Even with lower data density, the costs for chemiluminescent detections⁴⁹ are unlikely to contribute significantly to the overall sequencing costs. Because the larger part of the expenses is for chemiluminescent substrates, costs could be reduced by using lower concentrations of substrate, or with colorimetric detection.

How do the biotin and digoxigenin systems and peroxidase vs phosphatase compare? Both labels have considerable advantages: biotinylated phosphoramidites are commercially available and make primer walking strategies easy, and we find it easier to obtain totally background-free patterns. The separate use of streptavidin and biotinylated alkaline phosphatase necessitates the use of an additional incubation and a few washing steps compared to digoxigenin; however, the use of streptavidin–phosphatase conjugates has been reported.⁵⁰

Digoxigenin offers detection protocols with fewer steps, and anti-digoxigenin–antibody conjugates with peroxidase or phosphatase can be used. Peroxidase-based enhanced chemiluminescence is sufficiently sensitive for normal sequencing; however, dioxetane substrates are more stable in solution and can give higher sensitivities at longer exposure times.

A convenient alternative to biotin- or digoxigenin-labeled primers is the use of oligonucleotides that are complementary to the primer and covalently cross-linked to alkaline phosphatase^{51,52}; this approach can also be used for multiplex sequencing.^{21,53}

⁴⁹ The cost of one chemiluminescent detection is about \$50 for a 30 × 40 cm membrane; with 24 clones and 400 readable bases per clone, the cost per base pair of raw data is well below one cent.

⁵⁰ C. Martin, L. Bresnick, R.-R. Juo, J. C. Voyta, and I. Bronstein, *BioTechniques* **11**, 110–113 (1991).

⁵¹ E. Jablonski, E. W. Moomaw, R. H. Tullis, and J. L. Ruth, *Nucleic Acids Res.* **14**, 6115 (1986).

⁵² A. Murakami, J. Tada, K. Yamagata, and J. Takano, *Nucleic Acids Res.* **17**, 5587 (1989).

⁵³ P. Richterich and G. M. Church, unpublished results, 1990.

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[15] Adenine-Specific DNA Chemical Sequencing Reaction

By BRENT L. IVERSON and PETER B. DERVAN

DNA sequence determination according to the method of Maxam and Gilbert utilizes base-specific chemical modification reactions followed by a workup that causes cleavage of the sugar-phosphate backbone at the site of the modified base.¹ Reactions have been reported that are capable of selective cleavage of DNA at G, G + A, A > G, A > C, C, C + T, and T residues.¹⁻³ We describe here a simple protocol specific for adenine (A) that may be a useful addition to current chemical sequencing reactions. Reaction of DNA with K_2PdCl_4 at pH 2.0 followed by heating in the presence of piperidine produces an A-specific DNA cleavage reaction.⁴ The K_2PdCl_4 reaction involves selective depurination at adenine, affording an excision reaction analogous to the other chemical DNA sequencing reactions.

Materials and Methods

Cleavage of DNA at Adenine with K_2PdCl_4

Potassium tetrachloropalladate(II), K_2PdCl_4 (Aldrich, Milwaukee, WI) is dissolved to a final concentration of 10 mM in a 100 mM HCl solution previously adjusted to pH 2.0 with NaOH. This solution (40 μ l) is added to 160 μ l of a solution containing the ³²P-labeled DNA fragment⁵ and 1 μ g of sonicated calf thymus DNA in distilled H₂O. The reaction is mixed and incubated at room temperature for 20 min and stopped by adding 50 μ l of a solution containing 1.5 M sodium acetate, 1.0 M 2-mercaptoethanol and

¹ A. Maxam and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 560 (1977).

² A. Maxam and W. Gilbert, this series, Vol. 65, p. 499.

³ T. Friedmann and D. M. Brown, *Nucleic Acids Res.* **5**, 615 (1978).

⁴ B. L. Iverson and P. B. Dervan, *Nucleic Acids Res.* **15**, 7823 (1987).

⁵ M. W. Van Dyke, R. P. Hertzberg, and P. B. Dervan, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5470 (1982).