

Improvements of DNA Sequencing Gels

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We describe three simple modifications of DNA sequencing gels which all result in improved oligonucleotide resolution as visualized by autoradiography. First, it was possible to reduce the thickness of the gel to 0.2 mm by using new gel molding techniques. Second, the gel could be dried without any distortions of its dimensions by prior binding of the gel to the surface of the glass plate. Third, a uniform high temperature was obtained in all parts of the gel during electrophoresis by replacing one of the glass plates with an inexpensive thermostating plate with circulating water. The use of this heating plate resulted in a straight band pattern all over the gel and also in the resolution of such bands which were not resolved in other electrophoresis systems.

The new and rapid DNA sequencing techniques involve the generation of a set of oligonucleotides, which have one end in common and the other end varying in length with a single nucleotide, and the subsequent separation of the oligonucleotides on denaturing polyacrylamide gels. The set of oligonucleotides is produced either by primed DNA synthesis on a single-stranded DNA template in the presence of chain elongation terminators (2',3'-dideoxyribonucleoside triphosphates) or by base specific cleavages of end-labeled restriction fragments of DNA (1,2). The extent of sequence information that can be obtained from a single experiment is usually limited only by the resolving capacity of the gel electrophoresis. As the pattern of radioactive oligonucleotides in the gel is visualized by autoradiography, there will be some spreading of the bands due to radiation scattering within the gel. This effect can be minimized by reducing the gel thickness as beautifully demonstrated by Sanger and Coulson (3) who introduced the use of thin (0.4 mm thick) acrylamide gels for DNA sequencing. Difficulties in pouring gels free of bubbles and in handling them after electrophoresis has hindered the use of even thinner gels.

Preparation and handling of very thin (≤ 0.2 mm) gels and their advantage in electrophoretic separations have recently been reported by us (6). Here we describe two simple ways to prepare 0.2-mm-thick DNA sequencing gels covalently linked to one of the glass plates, thus facilitating gel drying after electrophoresis. The use of 0.2-mm-thick gels and their novel straightforward drying procedure are shown to improve the quality of the sequencing gels.

In order to ensure a uniform and constant temperature (up to 70°C) throughout the gel during electrophoresis, one of the two glass plates supporting the gel was replaced with a thermostating water-heated plate. The elevated temperature permits the resolution of oligonucleotides that are compressed together in conventional gel electrophoresis systems. By avoiding temperature differences within the gel, the thermostating plate creates equal electrophoresis conditions for all samples applied to the gel. Therefore the resulting autoradiograph shows straight bands in all parts of the gel.

MATERIALS AND METHODS

Materials. Acrylamide was from Eastman Kodak, *N,N'*-methylenebisacrylamide from

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Serva, and urea (ultra pure) was from Schwarz-Mann. Repelcoat was obtained from Hopkin & Williams Ltd., Rumford, England, and γ -methacryloxypropyl-trimethoxy silane from Wacker Chemie, München, West Germany.

The electrophoresis apparatus was essentially as described in (4), and all buffers and solutions as described in (5). The gels were formed between two glass plates ($200 \times 400 \times 4$ mm) one of which had a notch (165×20 mm) cut out from the top to allow contact between the gel and the buffer of the upper electrophoresis chamber. All glass plates used were cut from floated glass. Spacers (900×15 mm) and the well formers for 4-mm-wide and 8-mm-deep slots were cut from the same piece of 0.2-mm-thick PTFE. The thermostating plate was glued together from two glass plates and spacers as illustrated in Fig. 1. The water was heated and circulated using a LKB 2209 Multi-temp. Application of samples into the slots was done with thin-walled glass capillaries

(inner diameter = 0.1 mm) for X-ray analyses (A. Müller, Glas- und Vakuumtechnik, Berlin, West Germany). An LKB 2103 unit (2000 V) power supply was used.

Treatment of glass plates. Before casting the gel the notched plate (the A plate) was treated with Repelcoat and the other (plate B in Fig. 2) was treated with the γ -methacryloxypropyl-trimethoxy silane. The silane, with its organic and inorganic groups, will bind the gel covalently to plate B, whereas the Repelcoat will repel the gel from plate A. The A plate was wet on one side (marked) with concentrated Repelcoat solution, dried in a fume hood for 30 min, and then washed with ethanol before use. For the silane treatment of plate B two solutions were prepared: one contained 0.5 ml silane in 100 ml ethanol, and the other 0.3 ml acetic acid in 3 ml water. The two solutions were mixed just before wetting plate B on one side (marked) with the mixture. The silane treatment including drying was carried out at room temperature. After drying for 3 min the plate B was washed with ethanol and thoroughly wiped with Kleenex paper to remove excess silane.

Gel molding using the sliding technique. In this procedure gel casting is done on an even support ($180 \times 1000 \times 50$ mm) as illustrated in Figs. 2a-f. The B plate, with the silane-treated side up, is placed on the support some 5 cm in front of a dummy plate and the 900-mm-long spacers are placed over both plates. They are fixed in place with tape at the bottom end of the dummy plate and at the support above the B plate (Fig. 2a). A 0.2-mm-thick piece of PTFE (170×40 mm) is fixed with tape on the support between the two plates so that it extends about 4 mm on the upper side of the B plate at the bottom end (Fig. 2a,b). This PTFE piece acts as a piston and prevents air bubbles from entering from below during gel casting and it is therefore important that no empty space is left between it and the spacers on each side (Fig. 2b). Even better is to use

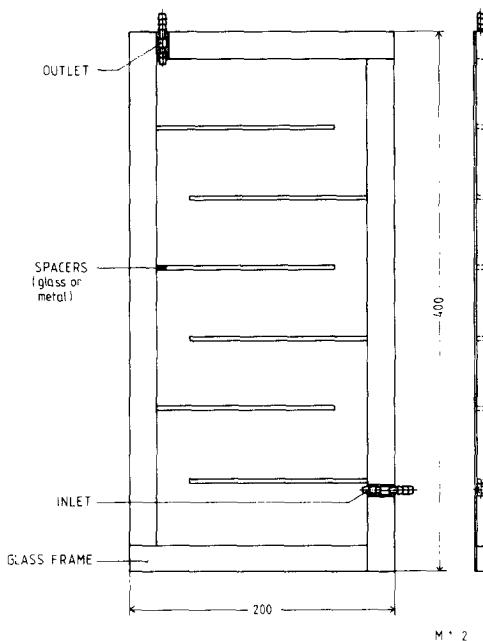


FIG. 1. Thermostating plate (schematically).

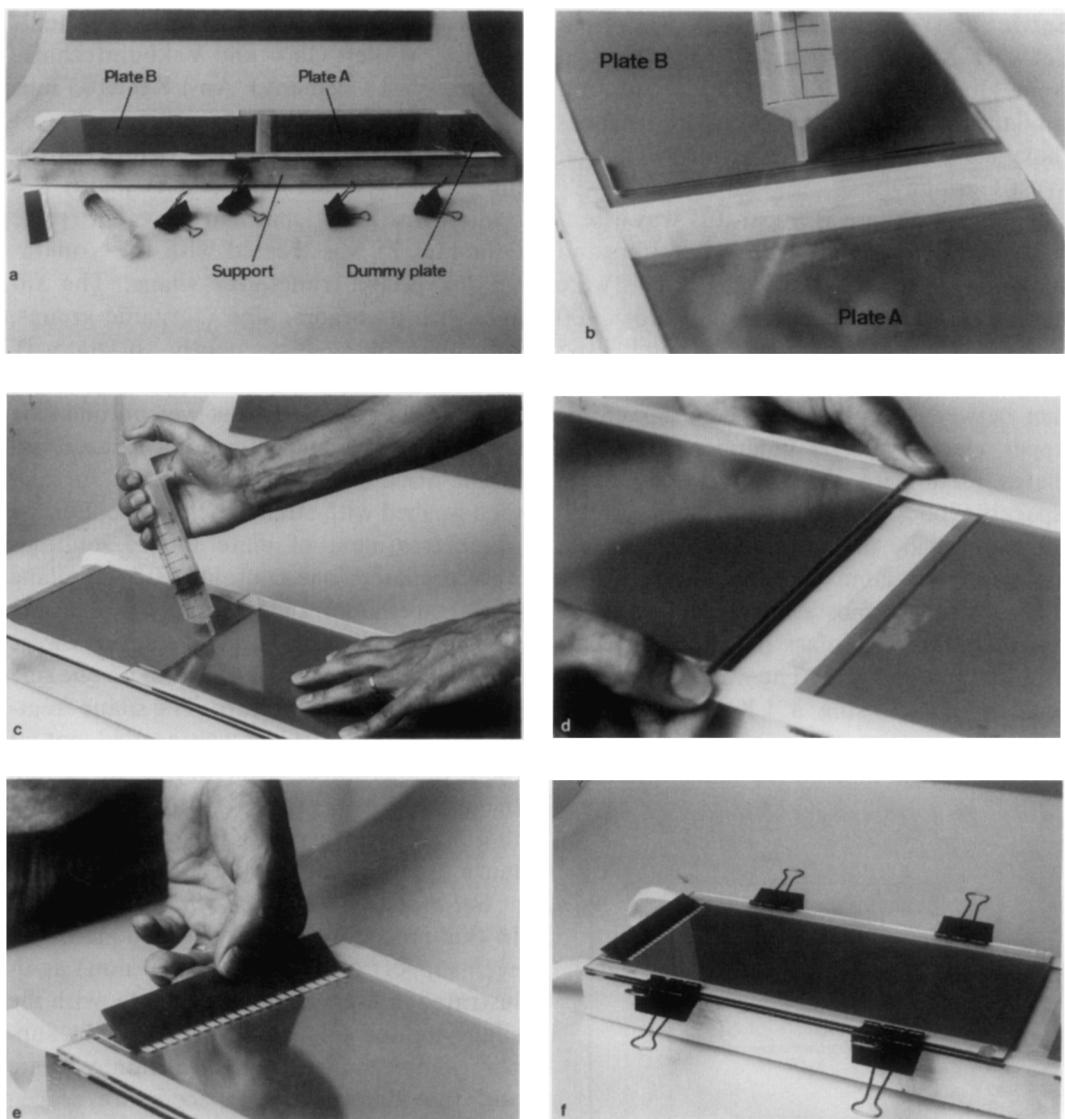


FIG. 2. Molding of a 0.2-mm-thick polyacrylamide gel using the sliding technique (for details see text).

a nylon net (Small Parts, Inc., Miami, Fla., part number CMN-37) for this purpose. In this case the nylon net is wider (about 220 mm) and is placed below the spacers. The A plate is placed on top of the dummy plate so that the Repelcoat-treated side is down and the notch is about 0.5 cm in front of the small PTFE piece on the B plate (Figs. 2a,b). The setup is now ready for gel casting.

Thirty milliliters acrylamide solution (5) is sucked into a syringe and then slowly poured in front of the notch letting the capillary force fill the space between the B and A plates when the latter is cautiously slid on top of the B plate (Figs. 2b,c). When the plates are completely superimposed (in 15–20 s) the small PTFE piece is removed from the gel (Fig. 2d). After the well former has

been inserted (Fig. 2e) the glass plates are clamped (Fig. 2f) and the gel is either left to polymerize on the support or removed in order to give place for the casting of a second gel.

For comparative analyses 0.4-mm-thick gels were also made by pouring the gel solution directly in between preassembled glass plates as described in (3).

When casting a 0.2-mm-thick gel between a thermostating plate and an A plate, the thermostating plate was placed on the support in front of a dummy plate with exactly the same thickness (e.g., a second thermostating plate). In this case the thermostating plate was treated with Repelcoat and the A plate with silane.

We have used the sliding technique as described above to prepare gels in the thickness range of 0.01–2 mm and up to about 1-m length. Even longer gels were prepared when the back edge of plate B was kept wet by continuous addition of gel solution through a perforated PVC tubing.

Gel casting using the piston technique. In this technique the B plate lies with its bottom end on the table and its top on a support so that it forms an angle of about 10° with the table. The spacers are spanned over the silanized upper glass surface and fixed in place with tape at the back side of the plate. A large (170 × 600 mm) 0.2-mm-thick piece of PTFE (the piston) is then put on the B plate between the spacers and on this the A plate. The A plate is prevented from sliding down by attaching its "ears" with tape to the B plate. The piston is put into starting position by drawing it from below until its upper edge is at level with the notch in the A plate. Gel molding is done by adding gel solution to the notch and slowly moving the piston downward. The gel solution follows smoothly the edge of the piston and when the piston is completely removed the whole space between the glass plates is filled with gel solution. After casting the gel the glass plates are lifted on a horizontal support, the

well former is inserted, and the plates are clamped.

To avoid bubbles in the gel it is important to let the top 2–3 cm of the piston become wet with gel solution before starting to move the piston downward. The speed of the downward motion of the piston should be adjusted so that the top edge of it always remains wet from gel solution.

Operation of thermostating plate. During electrophoresis at 2000 V the power dissipated on one plate (400 × 200 mm) was limited to 20–30 W, while during preelectrophoresis the power can be as high as 100 W. The plates were tested at 10,000 V potential difference across the gel and electrode buffers, without failure. The water was circulated through the plate under pressure of 160–300 mbar, as delivered by the thermostat pump. The plate was warmed up slowly at a rate of about 2°C/min during preelectrophoresis to the desired temperature. Thermal shock should be avoided.

Electrophoresis. The glass plates with the polymerized gel were mounted on the electrophoresis apparatus and the chambers were filled with electrode buffer before the well former was removed. Unpolymerized gel solution was carefully washed out from the wells through injection of a buffer stream with a Pasteur pipet. This had to be done immediately after removing the well former. After preelectrophoresis at 2000 V for 60 min, about 1.5 µl of a DNA sample in formamide-dye mixture (5) was loaded into the wells. The DNA samples were 5'-³²P end-labeled subclones from PBR-SFV 2 or the 947 bp EcoR1-XbaI fragment from PBR-SFV 3 (7,8) that had been subjected to base-specific cleavage reactions (G, A + G, C + T, C, C + A) as described by Maxam and Gilbert (2). Electrophoresis was done at maximum voltage available (2000 V), or, as indicated in the legends to Figs. 3–6.

Processing of gel after electrophoresis. After the electrophoresis the plates were separated with the aid of a spatula inserted into

a groove that had been milled into the bottom end of the A plate. The gel adhering to the silane-treated plate was fixed in 10% acetic acid for 15 min at room temperature (agitation was not necessary), blotted carefully with lint-free paper, dried for about an hour at 80°C, and autoradiographed for 1–7 days at –30°C, using Kodak X-Omat film and an intensifying screen (Dupont, Cronex Lighting Plus) (9,10). Autoradiography without the screen was done at room temperature.

Removal of the covalently bound gel from a glass plate. The glass plate with bound gel was placed overnight in 10% Deconex (Borer Chemie AG, Solothurn, Switzerland) solution (or 1/2 h in concentrated Deconex) and then brushed under tap water. Before reuse it was further washed with deionized water and finally with ethanol.

RESULTS

Separation of Oligonucleotides on 0.2-mm-Thick Gels

The separation of DNA molecules on the 0.2-mm-thick gels was clearly better than that which had been obtained earlier on 0.4-mm-thick gels in our laboratory. This is shown in Fig. 3 where the same oligonucleotide mixture has been analyzed on 0.4-mm and 0.2-mm-thick gels. In both cases the film was exposed to the wet gel covered with a polyethylene foil. The bands are much sharper on the thinner gel and many more bases can be read from this gel than from the thicker one.

The Effect of Gel Drying on Band Sharpness in the Autoradiogram

A DNA sample was run on two 0.2-mm-thick gels under identical conditions, and one of the gels was fixed and dried before autoradiography. The autoradiogram of the dried gel (Fig. 4, right) showed much sharper and better resolved bands than that of the wet gel covered with a polyethylene foil (Fig. 4, left).

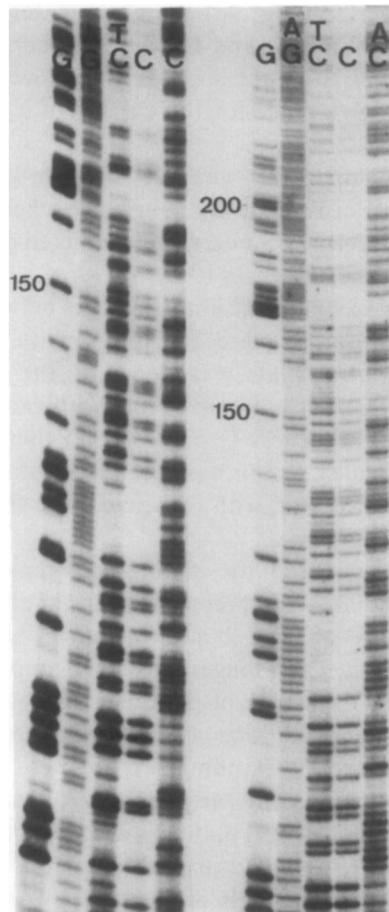


FIG. 3. Separation of the same sets of oligonucleotides on a 0.4-mm (left) and a 0.2-mm (right)-thick 8% acrylamide gel. The sample was end-labeled subclone 3 of PBR-SFV2 cleaved at various bases which are indicated at the top of the gel using the Maxam and Gilbert cleavage reactions. Three microliters of sample was applied into the slots of the 0.4-mm-thick gel and 1.5 μ l into those of the thinner gel. Electrophoresis was performed using a constant power of about 50 W (1800 V) for the thicker gel and about 25 W (2000 V) for the thinner one. The thin gel was run for 125 min and the thick one for 180 min after which the A plate was removed and the gels were covered with polyethylene film for autoradiography with screen, at –30°C for 3 days. The number 150 indicates the base number from the labeled end.

The Usefulness of a Thermostating Plate

In our earlier sequencing of the Semliki Forest virus (SFV) genome (7,8) using 0.4-mm-thick gels as described in (4) we en-

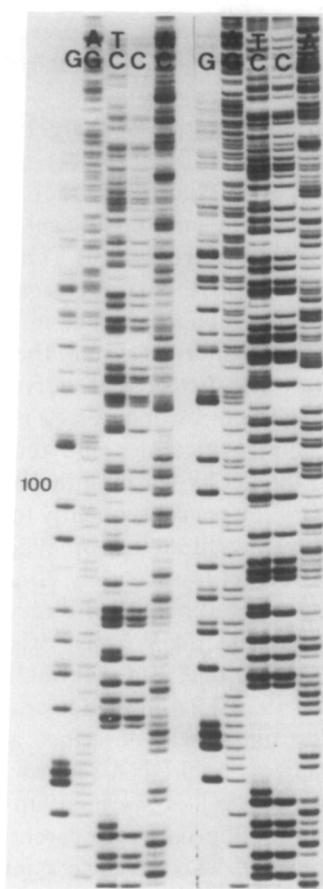


FIG. 4. The effect of drying of the gel on autoradiography. The same sample has been analyzed on two 0.2-mm-thick 8% acrylamide gels at 25 W for 90 min. The gel shown at the left was subjected to autoradiography as wet for 3 days with a screen, and the right one was first fixed, dried in an oven for 120 min, and then autoradiographed also for 3 days with a screen. The sample was end-labeled subclone 4 of PBR-SFV-2 cleaved at the various bases indicated at the top of the gel.

countered difficulties in reading the bases 75–116 from a 947 bp *EcoRI-XbaI* fragment labeled at the *EcoRI* site. The gel showed in this region severe compressions and gaps (Fig. 5, right). When the same sample was run at high temperature (70°C) using the thermostating plate the sequence C-G-C-C-C-G-C-G-C-C-C-G-G-C-G-G-C-C-G-T-C-C-T-T-G-G-C-C-G-T-T-G-C-A-G-G-C-C-A-C-T

could be read without difficulties (Fig. 5, left). The use of the high temperature does not increase diffusion of the bands, as shown

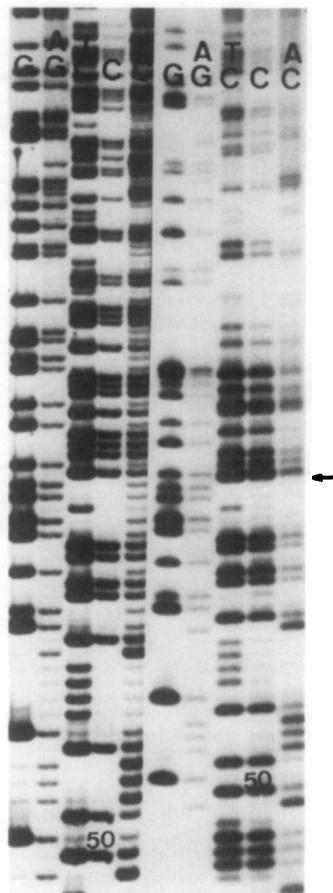


FIG. 5. Sample *EcoRI-XbaI* from clone PBR-SFV-3 analyzed for base sequence on a 0.4-mm-thick 8% acrylamide gel (right) and on a 0.2-mm-thick acrylamide gel with a thermostating plate. A constant power of 25 W (2000 V) was used during electrophoresis on the 0.2-mm-thick gel and about 50 W (1800 V) when running the 0.4-mm-thick gel. The running time was about 120 min for the thicker gel and about 80 min for the thinner gel. The measured temperature in the middle of the 0.4-mm gel was about 50°C during the run, whereas the 0.2-mm gel was kept at 70°C with the aid of warm water circulating through the thermostating plate. The thicker gel has been autoradiographed wet, covered with polyethylene foil for 4 days with a screen, and the thinner one has been fixed and dried before autoradiography, also for 4 days with a screen. The arrow indicates the beginning of the compressed region on the 0.4-mm gel.

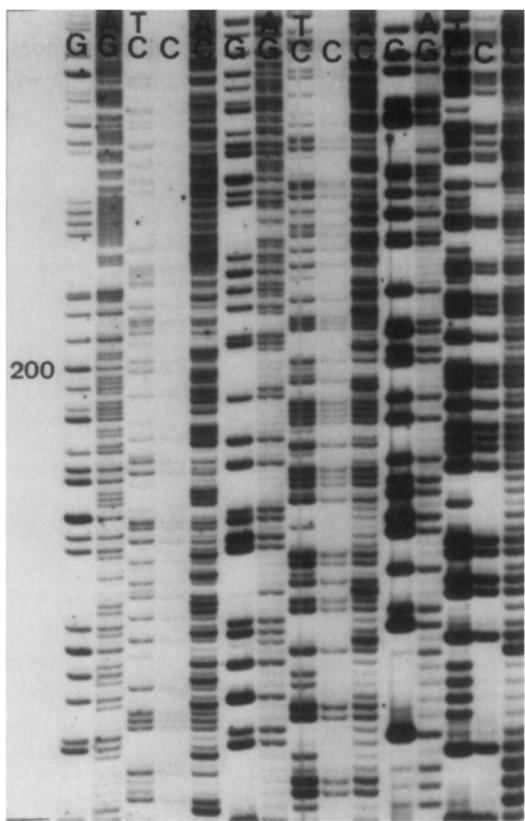


FIG. 6. The same sample as in Fig. 5, run for 90 (right), 120 (center), and 160 min (left).

in Fig. 6 (top left) where oligonucleotides containing much over 200 bases are still seen as sharp and well resolved bands. Figure 6 also shows that the use of the thermostating plate results in vertically and horizontally straight band patterns. There is no bending of the off-center bands ("smiling" effect) which is often a problem in gels run without a thermostating plate.

DISCUSSION

The sliding and the piston technique for casting thin gels are both very rapid and provided that the gel solution is kept on ice, several gels can easily be molded after each other using the same gel solution. Gels thinner than 0.2 mm have been casted with the

sliding technique and we are presently testing the resolution of oligonucleotides on these gels.

The covalent fixation of the gel to one of the glass plates with the aid of γ -methacryloxypropyl-trimethoxy silane offers several advantages in handling the gel both during and after electrophoresis. The sample slots are stable in their dimensions during the whole electrophoresis run; in the conventional electrophoresis system the bottoms of the slots may distort with curved bands as a result. After electrophoresis it is possible to fix the oligonucleotides and remove the urea from the gel by washing in 10% acetic acid, and dry the gel subsequently in an oven at 80°C for 1 h without any breakage of the gel. The dried gel layer is only 20 μm thick and can be autoradiographed directly without a polyethylene foil. We have dried in this way 4, 6, 8, 12, and 20% acrylamide gels. Gels thicker than 0.2 mm tend to crack when dried in the simple way described above.

In the conventional DNA sequencing procedure (3) the gel is warmed up by the joule's heat developed by the current passing through the gel. This leads to nonuniform temperature distribution within the gel which, in turn, causes thermal distortions in the band pattern, and sometimes even cracking of the glass plates. These inconveniences can be avoided by separating the gel heating function from the applied field. We have achieved this by constructing a simple thermostating plate with water circulation from a thermostat. Thermostating plates have been described and used before in gel electrophoresis. When compared, the present plate has the following advantages: (1) It is significantly less expensive due to choice of materials, simple form of the parts, and the easy way in which they are assembled. (2) The operational temperature ranges from well below 0°C up to +90°C. (3) The gel is cast directly between the thermostating plate and a glass cover plate. In this way the heat transfer and temperature uniformity

are improved. (4) It is transparent. Loading of the samples and migration of marker dyes can be followed.

We have tried to equalize the temperature differences (and straighten the bands) in the gel using several other means instead of the thermostating plate. Some straightening of the band pattern in the gel was observed when aluminum plates were clamped on the outside of the glass plates or when the air convection was increased by using a ventilator. Very straight bands were observed when the electrophoresis run was performed in the presence of a 3- to 4-mm water jacket on the outer surface of the B plate. The water jacket was kept in place by clamping a third glass plate and appropriate rubber tube sealing onto the B plate. None of the procedures above do, however, provide the gel with any extraneous heat as does the thermostating plate and they therefore tend to cool down the thin gel. This might lead to difficulties in resolving oligonucleotides that are hard to denature completely (e.g., the G-C rich region of the 947 bp *EcoRI-XbaI* fragment described above). The thermostating plate was found to be the only way by which a constant high temperature was assured in all parts of the gel during electrophoresis.

The modifications of the DNA sequencing gels described above (gel thickness 0.2 mm, covalent attachment of the gel to the glass, and the use of the thermostating plate) all contributed to an improved resolution of oligonucleotides in the gel. Recent experiments with samples containing more radioactivity have demonstrated that further improvement of the band resolution in the autoradiogram is achieved when the intensifying screen is omitted during autoradiography. Using 6% acrylamide we have lately been consistently reading 250–300 nucleotides from end-labeled samples using these modifications. We expect that even more bases can be read if the sample is run for a very long distance, e.g., on a 900-mm-long gel to maximize the separation of the larger oli-

gonucleotides. Such long gels (0.2 mm thick) have been cast on a thermostating plate using the sliding technique.

Obviously the sequencing result is much dependent on the quality of the sample. In our present work we have only analyzed end-labeled DNA samples that have been subjected to the base-specific cleavage reactions described by Maxam and Gilbert (2). Although we have very limited experience with samples produced by the chain terminator technique, these appear to give even better oligonucleotide resolution. This might be due to the facts that very few manipulations are needed to produce the samples and that these usually contain more radioactivity and display a more even size distribution than the samples made by base-specific cleavages.

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