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Rapid DNA sequencing by horizontal ultrathin gel electrophoresis

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ABSTRACT

A horizontal polyacrylamide gel electrophoresis apparatus has been developed that decreases the time required to separate the DNA fragments produced in enzymatic sequencing reactions. The configuration of this apparatus and the use of circulating coolant directly under the glass plates result in heat exchange that is approximately nine times more efficient than passive thermal transfer methods commonly used. Bubble-free gels as thin as 25μm can be routinely cast on this device. The application to these ultrathin gels of electric fields up to 250 volts/cm permits the rapid separation of multiple DNA sequencing reactions in parallel. When used in conjunction with ^{32}P-based autoradiography, the DNA bands appear substantially sharper than those obtained in conventional electrophoresis. This increased sharpness permits shorter autoradiographic exposure times and longer sequence reads.

INTRODUCTION

DNA sequence analysis remains a pivotal technology in molecular biology research. Over fifty million bases of sequence data have been published to date, and the international databases storing the information continue to grow exponentially. Although automated sequencing instruments have been developed which address different aspects of the sequencing process, the success of the Human Genome Initiative and other large-scale sequencing efforts clearly require the development of more powerful and inexpensive methods.

Existing methods for DNA sequence analysis rely on the separation of DNA fragments by polyacrylamide gel electrophoresis. The throughput of instruments which automate the separation and detection steps is limited by the speed of this process. Recently we and others (1–4) have shown that it is possible to greatly increase the speed of the electrophoretic separation by using capillary gel electrophoresis. In this method the fluorescently labeled products of enzymatic sequencing reactions are separated and detected in 50μm diameter polyacrylamide gels cast in fused silica capillaries. The high efficiency of heat dissipation in these capillaries permits much larger electric fields to be applied without damage from heating, yielding a concomitant increase in the speed of the electrophoretic separations. Speeds have been increased by up to 25 times over conventional methods in this approach.

Although the capillary electrophoretic methods do give a high separation speed, the overall throughput of sequence analysis by this method is no greater than that of commercial fluorescence-based automated DNA sequencers which are able to analyze up to 24 samples in parallel, albeit at a lower speed per sample. In order to exploit the increased speed of the capillary methods, it is thus essential to develop the capability to analyze many samples in parallel. There are two obvious approaches to this problem: 1) employ many gel-filled capillaries in parallel (5), or 2) perform the separations in a slab gel format (6). The former alternative presents a number of practical problems related to their cost, difficulties in their preparation, alignment issues, and so on. We have chosen therefore to explore the latter option.

This manuscript describes the design and use of an ultrathin slab gel electrophoresis apparatus in which high speed separations of up to 24 sequencing reactions (from six template DNAs) are performed in parallel. Approximately 250 bases of sequence data per template can be resolved in a fifteen minute electrophoresis. The sample well design and the method of sample introduction make the well dimensions independent of gel thickness. Samples are readily applied to horizontal gels as thin as 25μm. The speed and ease of use of this horizontal system suggests that it should have considerable utility in both radioactive and fluorescence-based sequencing methods.

MATERIALS AND METHODS

Apparatus construction and components

Schematic diagrams of the apparatus are shown in Figures 1A-C. The base of the apparatus (3.5×18.0×34.0cm) is composed of polycarbonate plastic and has been machined to provide space for a series of clamps, guide blocks, electrode supports, a water jacket and inlet and outlet manifolds.

Temperature regulation is provided by circulating coolant from a water bath (VWR Scientific, Model #1140, Chicago, IL) through the water jacket under the glass plates. Fluid flow is dispersed across the width of the glass by the inlet and outlet manifolds, which mix the fluid in the water jacket and thus aid in obtaining a uniform temperature distribution across the glass plates.

Correct alignment of the glass components on the apparatus is provided by the end alignment bar/electrode support assembly and two guide blocks. These aid in the correct positioning of the glass components on the water jacket o-ring. In addition, pressure-adjustment screws on the end alignment bar apply

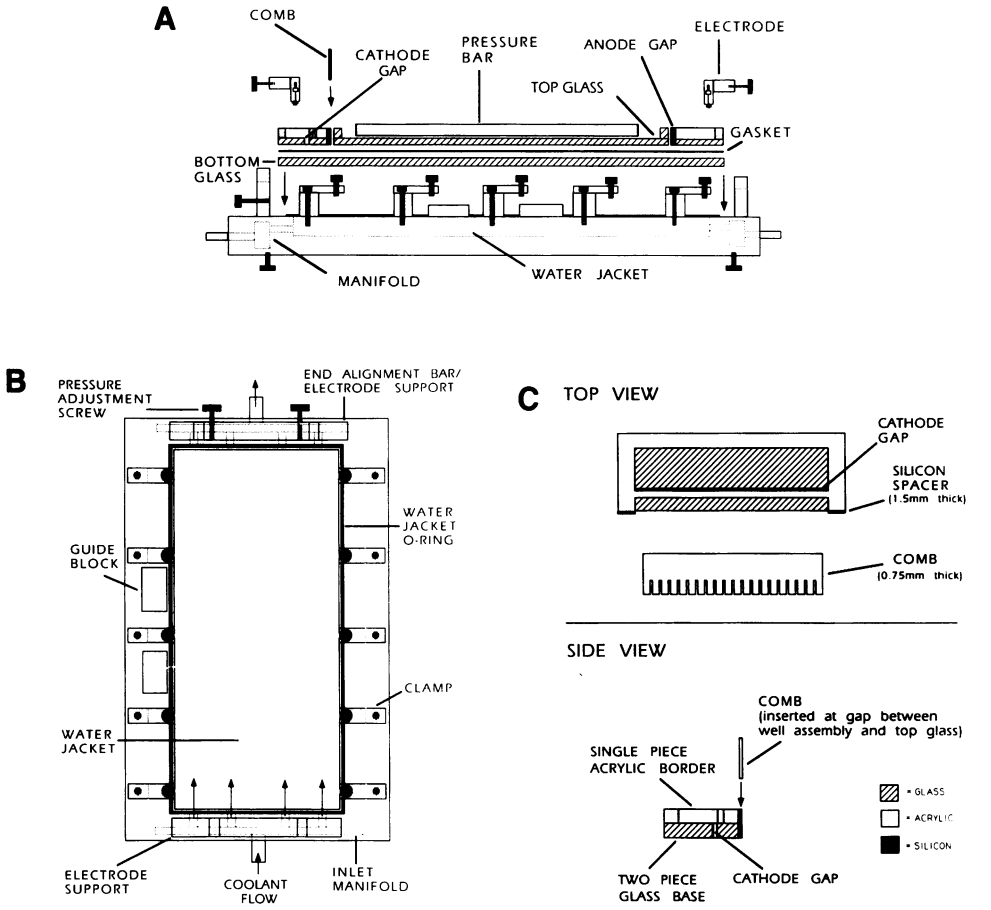

Figure 1. A) Side view of horizontal apparatus base and glass components; B) Top view of horizontal apparatus base; C) expanded view of Buffer Chamber/Sample Well Assembly. Further technical information useful in the construction of the apparatus will be provided upon request.

pressure at the end of the buffer chamber/well assembly. This ensures that the comb fits properly between the glass components and is essential for the consistent formation of sample wells.

Electrodes were constructed by gluing graphite rods into a plastic support (Figure 1A). This electrode assembly contains two height adjustment screws and a 'banana' connector that provides contact between the graphite electrode and the power supply. Electrical contact to the gel prior to electrophoresis is accomplished by placing the adjustment screws into slots in the electrode supports. The graphite electrodes are then placed in contact with the buffer and the assemblies are secured by tightening the height adjustment screws.

The buffer chamber and buffer chamber/well assembly (Figure 2) were designed to hold up to 5ml of liquid buffer as well as to serve several other functions. When clamped into place, these chambers prevent the occurrence of leaks during gel pouring by providing a liquid tight seal at each end of the assembled glass plates. Figure 2 shows the slots formed by the juxtaposition of the top glass with the silicon spacers on the buffer chamber (anode gap) and the slot in the buffer chamber/sample well assembly (cathode gap) which provide electrical contact between the gel and the buffer. The buffer chamber/well assembly provides a small chamber for rinsing the wells prior to sample loading and also has silicon spacers which form a gap for the comb.

The glass components, comb and gasket are also shown in Figure 2. The comb is made of 0.75mm high-density polyethylene, and is 3.8×7.2cm with 0.5mm slots and 2.5 or 5.0mm

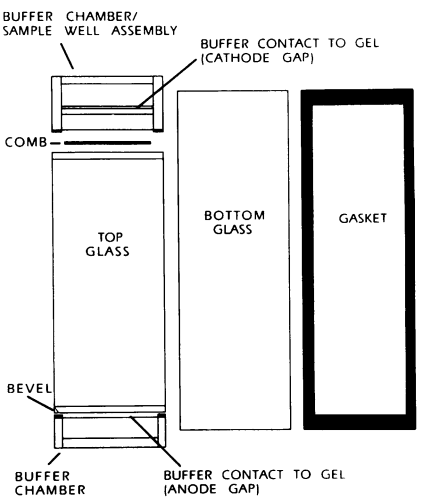

Figure 2. Top view of glass and plastic components.

wells. The bottom glass plate is 10.0×30.5×0.5cm, whereas the top glass plate is 10.0×25.0×0.5cm. To ensure uniformity of glass surfaces, optical quality (BK-7, Tempax) or soda lime glass polished to four wave (~2μm) flatness over any two inch surface, and cut with opposite sides parallel (obtained from American Precision Glass, Duryea, PA) was employed. The glass

face of the buffer chamber/well assembly and end of the top glass that form the gap for the comb were polished flat to within 5μm. The gasket, which determines the thickness of the gel, is cut from a polyester sheet (Acry Fab, Inc, Sun Prairie, WI) of the desired thickness. The final gel area was 205 cm^2 (8.2 cm×25 cm).

Apparatus assembly and gel pouring

The preparation of glass components used in this apparatus is essentially the same as conventional slab gels. All glass pieces are washed with a mild cleanser, rinsed with deionized water and thoroughly cleaned with three ethanol wipes. Since the gels are fixed and dried directly on the glass after electrophoresis, the bottom glass is treated with γ-methacryloxypropyltrimethoxysilane to bond the polyacrylamide to the glass surface (7). Likewise, the end of the top plate where the comb is placed is treated to prevent distortion of the divisions between sample wells. The top glass can be siliconized (7) to aid in the uniform flow of polyacrylamide solution during gel pouring.

The correct orientation of components on the apparatus is shown in Figure 1a and are assembled as follows: The bottom glass is positioned on the apparatus using the guide blocks and end alignment bar. The gel gasket is then placed on the bottom glass and properly oriented using the same guides. The spacers of the buffer chamber/well assembly and buffer chamber are coated with a small amount of petroleum jelly to ensure a proper seal with the top glass plate. The buffer chamber/well assembly, top glass plate and buffer chamber are then positioned on the bottom glass. The clamps over the pressure bars on the top glass and on the buffer chamber are securely tightened, while the clamps over the buffer chamber/well assembly are tightened just enough to allow for subsequent positioning by the pressure adjustment screws. Once the components are assembled, the apparatus is elevated slightly at one end (typically the end closest to the sample wells), placing it at about a 15 degree angle.

Gels are prepared by mixing 10mls 6% polyacrylamide with 50μl 10% APS and 5μl TEMED. Approximately 5mls of solution are poured into the elevated buffer chamber, and the gel flows to the lower chamber. Although the gel solution usually flows evenly across the width of the glass, dust or small imperfections on the glass may retard the flow, potentially causing bubbles to form. This can be avoided by observing the solution as it flows, and gently tapping the top glass at points that appear to be moving slower than the solution front. To complete gel pouring, one ml of solution is poured into the lower chamber when the solution front reaches the end of the top glass. The apparatus is then placed level on the lab bench, and the comb is inserted in the slot between the top glass and well assembly such that the 'teeth' of the comb rest on the bottom glass. The pressure adjustment screws are then tightened just enough to eliminate gel solution from between the comb and the top glass, thus inhibiting the formation of polyacrylamide 'curtains' in the sample wells. Gels are allowed to polymerize for at least 90 min before use.

Sequencing reactions

Sequencing reactions with Bst DNA polymerase were performed essentially as described previously (8) with the following modifications. Briefly, 1.0pmol ^{32}P (5000 Ci/mmol) end-labelled primer is combined with 0.8pmol M13mp19 template in 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.5 to a final volume of 10μl. The primer-template mixture is incubated at 65°C for 2 min and allowed to anneal at room temperature for 5 min. Bst DNA polymerase (Bio-Rad laboratories, Richmond, CA) is added

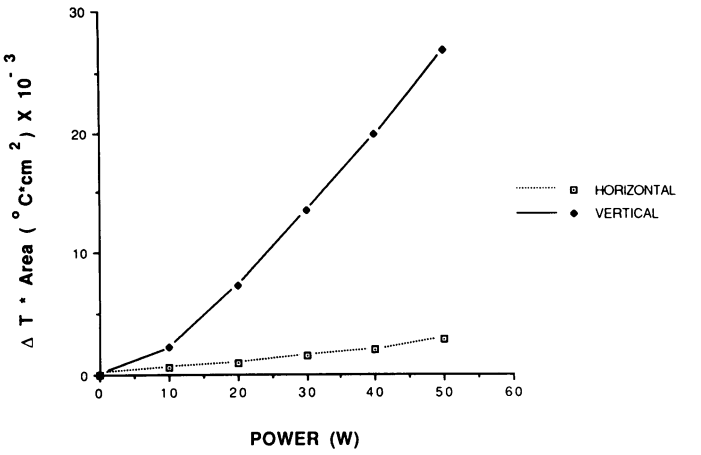

Figure 3. A plot of temperature gain corrected for area (ΔT * Area) versus applied power (W). The steeper slope obtained with the vertical apparatus indicates less efficient heat transfer.

to a final concentration of 0.05 U/μl and 2.5μl of the final mixture is added to 2.0μl of each d/ddNTP mix. The sequencing reactions are incubated at 65°C for 5 min and stopped with 2.0μl of stop solution (95% deionized formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). Samples are denatured at 70°C for a minimum of 2 min before loading.

Sample loading and electrophoresis

Prior to electrophoresis, water is preheated to the desired temperature (typically 35°C), and circulated through the water jacket for about 5 min to allow the glass plates to reach a uniform temperature. Excess unpolymerized polyacrylamide is blotted from the buffer chambers followed by the addition of 5mls of electrophoresis buffer (1×TBE; 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.3) to each chamber. About 1 ml of distilled water is added to the sample chamber, and wells are rinsed by gently pipetting 100μl of fluid into each well (3–4 times/well). After rinsing, excess water is removed from the sample chamber by blotting with an absorbant paper towel and samples are loaded using a 1.0μl Hamilton syringe. The low conductivity of the water overlay channels the electrophoresis current through the sample, producing a rapid injection. The resolution of the DNA bands is significantly lower if a higher conductivity buffer overlay is employed (data not shown). The graphite electrodes are positioned as described earlier and gels are electrophoresed at constant power (40W; 150–250 V/cm) using a 6.25 kV power supply (E-C Apparatus Inc., Model #EC-650, St. Petersburg, FL). After 30 sec, electrophoresis is interrupted just long enough to replace the distilled water in the sample chamber with 2mls 1×TBE. We have found that this step is necessary to avoid subsequent breakdown of the gel. For safety considerations, the entire apparatus is enclosed in a transparent plastic shield equipped with a safety interlock switch that interrupts the current from the power supply when the lid is opened.

Once electrophoresis is complete, the water bath is turned off and the water jacket is emptied by allowing the fluid to drain into the bath. The electrodes are disconnected from the apparatus and the buffer is discarded (the buffer in the anodic chamber is disposed of in radioactive waste). The clamps are then loosened,

the buffer chambers removed, and any contaminated gel adhering to the glass is placed in radioactive waste. The top and bottom glass plates are separated by inserting a razor blade at the beveled corner of the top glass and gently prying the two pieces apart. The gel is then fixed with a 10% methanol, 10% acetic acid solution for about 2 min and rinsed thoroughly with distilled water. Gels are dried by placing the bottom glass on an 45×40cm aluminum plate/hot plate at 60−70°C for 10 min. Autoradiography is performed by exposing gels to X-ray film at room temperature for the times indicated (Figures 4−5).

Vertical gel electrophoresis

All methods for gel fixing/drying, autoradiography and reagents used for vertical gels were identical to those used for the horizontal gels described above. Briefly, 6% polyacrylamide gels were cast using 0.25mm combs and spacers and allowed to polymerize for 90 min before use. Electrophoresis was performed on an adjustable nucleic acid sequencer (American Bioanalytical, Model # SU-1000-33, Natick, MA) and passive heat transfer was provided by clamping an aluminum plate to the glass. Gels were prerun for approximately 30 min and samples electrophoresed at 40W constant power for the times indicated in Figure 4. Gel plates were 33×40 cm, and the final gel area was 1428 cm^2 (33.2 cm×43cm).

Temperature measurements

Measurements of temperature gain per unit applied power for vertical and horizontal electrophoresis were performed as follows: a thermocouple with digital output (Fluke 51K/J thermometer, Everett, WA) was taped to the center of the front glass (vertical) or top glass (horizontal). For each apparatus the applied power

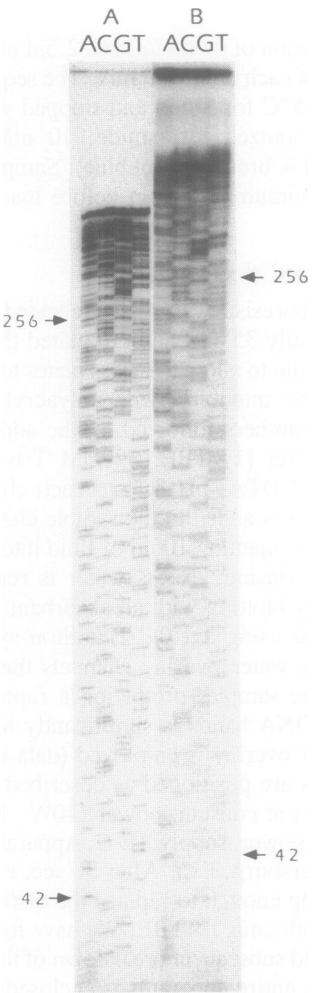

Figure 4. Comparison of sequence generated on a horizontal apparatus (A) and a vertical apparatus (B). An equivalent volume of sample (0.5μl) from the same sequencing reaction (see Methods) was loaded in each lane. Both gels were dried directly on the glass plates and exposed to X-ray film for 2.5 hr. The horizontal gel (50μm thick) was electrophoresed at 40W constant power for 16min. (134−222V/cm). The vertical gel (0.25mm thick) was electrophoresed at 40W constant power for 71min. (37.7−55.8V/cm). The bromophenol blue dye marker was migrated 25.0 cm from the sample well in both gels.

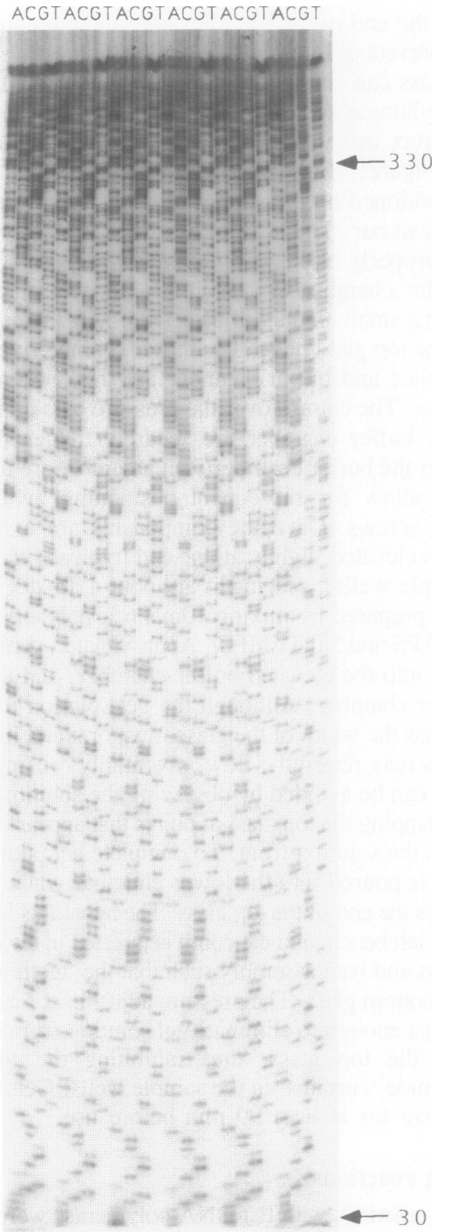

Figure 5. Autoradiograph obtained on the horizontal apparatus with six sets of sequencing reactions run in parallel. The gel was electrophoresed for 17min at 40W constant power (128−213V/cm). The sample wells are 2.5mm wide with 0.5mm divisions between wells. The gel is 8.25cm wide and 25.0cm long. 180nl of each sequencing reaction (see Methods) were loaded per well. Samples were loaded to within 0.5cm of the gel gasket.

was changed from 0−50W in increments of 10W. The temperature was allowed to stabilize before being recorded. The values of ΔT plotted in Figure 3 represent the difference between the surface temperature measured at a given applied power and the initial surface temperature measured prior to application of the electric field.

RESULTS

The heat transfer characteristics of the horizontal and vertical systems are shown in Figure 3. This plot shows the measured temperature gain corrected for area as a function of the applied power. The difference in surface temperature between the two systems corresponds to an approximately nine fold more efficient heat transfer in the horizontal than in the vertical system.

A comparison of DNA sequence data obtained in vertical (250μm thick gel) and horizontal (50μm gel) electrophoresis is shown in Figure 4. Each autoradiograph was read by two individuals with different levels of sequencing experience. The less experienced reader was able to call 108 bases (99.1% accurate) from the vertical gel and 177 bases (99.4% accurate) from the horizontal gel. The other individual read 154 bases (98.7% accurate) from the vertical gel and 272 bases (98.9% accurate) from the horizontal gel. In both cases, the sequence collected from the vertical gel was less than 2/3 the amount of that obtained from the horizontal gel, presumably due to the greater band sharpness seen in the horizontal gel. Although the reasons for this increased band sharpness have not been investigated in detail, one major factor is likely to be the decreased thickness of the radioactive sample being detected on the X-ray film. A 50μm thick gel, after drying, is only about 3μm thick, giving close to a point source in the autoradiographic imaging process. The increased sharpness of the bands evident in Figure 4 also permits the autoradiographic exposure time to be reduced by about two-fold with good results.

The data shown in Figure 4 also show a difference in GC compressions. Whereas GC compressions are evident at bases 34, 95, and 179 in the vertical gel, these compressions are absent in the horizontal gel. This observation is presumably related to the difference in running temperatures between the two gels. The horizontal gel was heated with the circulating coolant to 35°C and then electrophoresed at 40W constant power, resulting in a final gel temperature of 45−50°C. Although the vertical gel was pre-electrophoresed (to heat the gel), the running temperature was only about 35°C. This lower temperature was apparently insufficient to melt secondary structures. Efforts to run the vertical gel at a higher power were in this case unsuccessful due to gel breakdown.

Figure 5 shows that good quality sequence data may be obtained from as many as six templates on a horizontal gel 7.5cm in width. We estimate that approximately 1.5 kb of raw sequence is contained on one gel with six sets of reactions (250 bases per set). The total time required to obtain this data, from glass preparation to data acquisition, was less than 5 hr. We have also used this apparatus to obtain 453 bases (98.0% accurate) of contiguous sequence from one M13 clone using one 6% (base 27 to 299) and one 5% (base 271 to 480) gel (data not shown).

DISCUSSION

In this manuscript we describe a new apparatus we have developed for ultra thin slab gel electrophoresis. Although methods for the preparation and use of ultrathin slab gels have been in the literature since 1980 (9,10), these thin gel systems have not to date found widepread utility, presumably due to practical problems in their use. One of the key difficulties in the use of ultrathin gels has been the problem of sample introduction. In vertical slab gel systems the dimensions of the sample wells are defined by the thickness of the gel; as the gel is made thinner, it becomes increasingly difficult to physically introduce a sample into the thin space available between the plates. This design constraint has limited the thickness of commonly used sequencing gels to 0.2mm or greater.

In the horizontal gel system described here the sample wells are introduced into the gel *perpendicular* to the direction of the electric field. Thus the sample well dimensions are independent of the gel thickness, making it much easier to load samples in very thin gels. The horizontal configuration also provides a substantial advantage related to thermal gradients in the gel system. In vertical gel systems as commonly employed, one of the two glass plate surfaces is exposed to the ambient air environment. Convective processes cause a thermal gradient on that surface, with a higher temperature at the top of the gel than at the bottom. This is a well known effect in convective heat tranfer, which may be quantified using Nusselt's equations (11), and is readily visualized by using thermochromic plastics (Davis Liquid Crystals, Inc., San Leandro, CA) as thermal indicators in contact with the glass surface. The horizontal configuration of the apparatus eliminates this convective temperature gradient, and thus permits higher powers to be applied to the gels without adverse effects (such as the cracking of glass plates due to thermal stress). The use of active temperature regulation with circulating coolant in direct contact with the lower gel plate further facilitates the use of higher powers by increasing the efficiency of heat transfer.

Thus there are three major aspects of thermal control operative in this system; 1) the gels themselves are very thin, thus producing less heat (for a given applied field the Joule or resistive heating decreases inversely with gel cross sectional area), 2) active temperature control is employed to regulate gel temperature, and 3) the horizontal configuration reduces convective temperature gradients. The plot in Figure 3 shows that the net result of these effects is a system with much better decoupling of temperature from the electrophoresis conditions than is found in conventional apparatus. It may be noted that only the last of these effects is specific to the horizontal configuration described here. It is therefore likely that much of the improved heat transfer obtained with this apparatus could also be realized in a suitably designed vertical system, assuming that an adequate sample introduction method was available.

There are two major benefits of this system; the increased electric field strength which may be applied without deleterious heat effects results in much more rapid separations of the DNA fragments, just as we have found in our previous capillary electrophoresis work; and the thinness of the gel gives increased resolution in sequence data obtained by autoradiography (see Figure 4). This latter effect allows one to read more sequence from a gel of a given length, a factor of considerable importance in sequencing projects. The increased band sharpness also permits an approximately two fold reduction in autoradiographic exposure time. It will be of interest to know if this apparent increase in resolution is solely a result of the autoradiographic imaging process, or in fact reflects a true increase in the resolution of the bands as they are present in the gel itself. We are in the process of constructing an apparatus for performing fluorescence-

based DNA sequence analysis in these gels which will allow this issue to be studied in detail.

These two effects together result in a sequencing system with excellent performance. The horizontal system yields a 77% increase in readable sequence per reaction obtained in 1/6 of the electrophoresis time. Single-base resolution of fragments up to 480 bases in length is obtained on a gel only 25cm in length. The gels themselves are easily and reproducibly prepared. The simple radioisotopic methods described in this paper will, we believe, have great utility in small scale sequencing as practiced in most research laboratories; and this electrophoresis apparatus used in conjunction with real-time fluorescence detection will constitute a very powerful automated sequencing system suitable for large scale projects.

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