Simple miniaturized gel system for DNA sequence analysis

Arnold Stein*, Stephanie A. Hill, Ziqiang Cheng and Minou Bina

ABSTRACT

A simple miniaturized gel system suitable for DNA sequencing is described. Small ultrathin polyacrylamide gels are cast, eight or more at a time, using standard microscope slides. Gels, ready to use, can be stored for ~2 weeks. Gels are run horizontally in a standard mini-agarose gel apparatus. Typical run times are 6–8 min. A novel sample loading system permits volumes of standard sequencing reactions as small as 0.1 µl to be analyzed. Sequencing ladders were visualized using 35S-labeled DNA by autoradiography and by colorimetric detection. Band resolution compares favorably with that of large gels. The methods introduced here serve as a step toward the miniaturization of DNA sequencing and are amenable to automated sample loading and detection.

INTRODUCTION

DNA sequence analysis has become an integral part of molecular biology, genetic and medical research. Although high throughput sequencing machines have been developed, most of the machines currently in use perform DNA fragment separations on standard, relatively large polyacrylamide gels. Further advancement of DNA sequencing technology will require the development of faster and less expensive methods.

Attempts to increase the speed of the electrophoretic separations required for DNA sequencing has resulted in the development of capillary gel electrophoresis (1,2) and ultrathin slab gels (3,4). In both of these methods the lower gel conductivity and greater heat dissipation allows much higher electric fields to be applied without damage to the gel by Joule heating. Electrophoresis times have been shortened ~20-fold, compared with conventional methods. Gel run times could be reduced further by miniaturization of the process. Miniaturization would also result in a substantial cost reduction, because of the small quantities of reagents and sample that would be required.

Although it has generally been thought that DNA sequencing gels need to be ≥40 cm long to achieve the single nucleotide resolution required, this is not necessarily true. Clearly one does not gain resolution by having relatively broad bands separated by large distances (as in conventional gels) compared with having very sharp bands separated by small distances. The image from a small gel can always be magnified for easier viewing and analysis.

Consistent with this idea, it has recently been demonstrated that DNA sequencing can be accomplished in gels contained in 50 x 8 µm cross-section channels microfabricated in a 2 x 3 inch glass ‘chip’ (5). Laser confocal fluorescence detection and four dye discrimination was used with instrumentation similar to that used in the Perkin Elmer/ABI machine. Although this system represents a major technological achievement and is the first published demonstration that DNA sequencing can be miniaturized, loading samples into the small capillaries electrophoretically was somewhat cumbersome. Moreover, in order to achieve adequate signal 1 µl of sample, containing ~100 times more template (per labeling reaction) than an ordinary sequencing reaction, was applied to each lane.

It seemed logical to us that the dimensions of a DNA sequencing gel could simply be reduced significantly while maintaining high resolution and using samples prepared by standard protocols if DNA sample volumes were reduced correspondingly and the samples could be loaded as sharp bands. Here a simple miniaturized gel system for manual DNA sequencing and analysis is described.

MATERIALS AND METHODS

Standard pre-cleaned 25 x 75 mm microscope slides (Gold Seal, Clay Adams) made of Swiss Glass were obtained from Baxter Scientific Products (McGaw Park, IL). Scotch 3M transparent tape was used for spacers. This tape was the thinnest tape (~50 µM) found commercially. The thickness was estimated by making a stack of 20 layers, which measured 1.0 mm. Single edged no. 9 razor blades made from surgical carbon steel were from VWR (Media, PA). Whatman 3 MM chromatography paper was used for wicks. Glass plates, 2 x 4 x 0.25 inch weighing ~74 g each (Lafayette Glass Co., Lafayette, IN), were used as weights on the polymerizing gels. All gel reagents were molecular biology grade. γ-Methacryloxypropyltrimethoxysilane was purchased from Sigma Chemical Co. (St. Louis, MO), Kodak MR Scientific Imaging Film was used for autoradiography. DNA sequencing reactions were performed using a USB 70770 Sequenase Version 2.0 DNA sequencing kit (US Biochemicals, Cleveland, OH) and [35S]dATP (ICN Pharmaceuticals Inc.). Colorimetric detection was performed using reagents from the New England Biolabs Phototope detection kit. 4-Nitroblue tetrazolium chloride (NBT)

*To whom correspondence should be addressed. Tel: +1 765 494 6546; Fax: +1 765 494 0876; Email: astein@bilbo.bio.purdue.edu
Figure 1. Gel design. Standard 25 × 75 mm microscope slides are used to make an ultrathin standard recipe DNA sequencing gel.

and X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were from Boehringer Mannheim.

Construction of the sample loading device

Razor sharp comb-like teeth, 0.023 wide × 0.098 inch deep, were fashioned (by W. Vaughn, Department of Chemistry, Purdue University) from a single edged razor blade by spark erosion using an electric discharge machine. Thus the bottom of each tooth maintained the sharp razor blade edge. To perform the spark erosion, 0.023 inch wide grooves were first cut into a brass electrode using a 0.023 inch wide slotting saw. Additionally, an aluminum holding fixture was made in order to hold the blade flat in a bench vice during the electrical discharge process.

Gel design and preparation

Gels are prepared using standard 25 × 75 mm microscope slides as plates. The bottom plates are treated with γ-methacryloxypropyltrimethoxysilane so that the polyacrylamide bonds to the glass (6). Two Scotch tape strips on the bottom plate, each 1/16 inch wide (Fig. 1a), serve as 50 µM thick spacers, resulting in a small ultrathin gel when a sandwich is made using the top plate. The top plates are scored using a tungsten carbide or diamond stylus on the inner face 0.5 inch from the end. A 0.5 inch extension is added to the other end of the bottom plate. The extension is cut from another microscope slide. The manufacturer’s edges are placed in contact and held together by a piece of tape placed on the outer face (Fig. 1b).

A novel procedure is used to quickly and easily ‘pour’ eight or more leak-free, bubble-free gels at one time. First, a standard recipe sequencing gel solution is prepared. For example, 100 ml of a solution consisting of 8.0% acrylamide (19:1 acrylamide:biacrylamide), 8.3 M urea and Tris–borate, EDTA (TBE) buffer is made. The solution is filtered and degassed, as usual. Ammonium persulfate (750 µl of a 10% solution) and TEMED (15 µl) are added to initiate polymerization and the solution is poured into a 150 × 25 mm polystyrene cell culture dish. Horizontal gel sandwiches are then quickly made while the plates are completely submerged under the gel forming solution. The amount of TEMED used should be such that polymerization occurs in ∼30 min. It generally takes only ∼2 min to make 10 gel sandwiches. Care is taken so that the submerged top and bottom plates are squarely aligned laterally. The extended top plate overhangs the bottom plate by 0.25 inch at each end. The sandwich is then withdrawn from the solution. Withdrawal is made easier by placing a microscope slide at the bottom of the dish as a platform (oriented at a 90° angle with respect to the gel plates), from which gel sandwiches can be more easily grasped.

After withdrawal from the solution a weight is placed upon each sandwich to apply even pressure during polymerization, resulting in a uniform ultrathin gel. Two stacked 2 × 4 × 0.25 inch, 74 g glass plates per gel are currently being used as weights. The polymerizing gels are left undisturbed for 1 h. Finally, the gels are cleaned up by scraping off any polyacrylamide formed on the outsides of the plates and by rinsing off crystallized urea. The clean clear gels (Fig. 1c) are inspected at this stage to see if any bubbles are present. Occasionally a bubble will be found and this gel will be rejected. The gels are then stored for later use in TBE, urea solution in a closed container. Gels have been successfully stored in this way for several weeks in the refrigerator.

Loading and running the gels

A gel is prepared for running by removing the top plate extension, exposing a portion of the gel for (right) wick contact and by snapping off the portion of the gel at the other end on the score line to expose the area for sample loading and (left) wick contact. The dry gel is then placed into an ordinary horizontal mini-agarose gel apparatus, for example BRL model H6. Wicks (2 × 5 cm) are wetted in gel storage buffer and placed in contact with the gel ends, as illustrated in Figure 2. Electrode buffer (1 × TBE) is added to each compartment to a level several centimeters below the gel, making contact with the wicks, and the gel is pre-run for 30 s at 1000 V. At this voltage a current of only ∼2 mA is usually obtained, which heats the gel to ∼40°C over the course of a 7 min run.

Figure 2. Gel with wicks in place, ready for loading, as on the platform of a standard horizontal gel apparatus.

Figure 3. Drawing of sample loading device, fashioned from a razor blade.
Before loading samples are heated for 5 min at 80°C and placed on ice, as usual. Then ~50 nl or less of each sample is deposited onto each tooth of the sample loading device (Fig. 3). This can be done by using a disposable micropipette tip to pick up ~100 nl by capillary action. A comb tooth conveniently fits into the end of a standard taper (yellow) tip and picks up a portion of the sample by surface tension. Alternatively, the optimum amount of sample (<50 nl) can be transferred to each tooth by stroking it with the loaded pipette tip one or more times. The comb teeth, evenly wetted with sample, are then stamped into the gel in the exposed area close to the edge of the top plate (Fig. 2). Sufficient pressure is used to cut completely through the gel. Using a smooth upward motion of the comb, the teeth are withdrawn from the gel, leaving separated razor sharp bands of loaded samples. The power supply is then switched on and electrophoresis begins. The power is turned off briefly at 2.5 min to adjust the left wick downward to cover the loading area and electrophoresis is then resumed. At 1000 V the bromophenol blue dye marker can be usually seen to enter the right-hand wick at ~6 min. At the end of the run the top plate is removed using a razor blade and discarded. The tape strips are removed from the bottom plate and the gel, supported by the bottom plate, is processed for autoradiography or non-radioactive detection.

Detection of the sequencing ladders

For autoradiographic detection (of 35S-labeled DNA) the gel, supported by the bottom plate, is soaked for 10 min in 10% methanol, 10% acetic acid, with gentle agitation. Next the gel is dried for 10 min on a heating block set at 75°C and exposed to X-ray film for 16 h.

Alternatively, the gel can be blotted and processed for colorimetric detection. For this type of detection a biotinylated sequencing primer is used in the sequencing reactions. The gel is run as before, then immediately blotted by placing the gel surface in direct contact with a piece of wet nylon hybridization membrane, upon which a piece of dry filter paper and a weight are placed. After 15 min the DNA bands become nearly quantitatively transferred to the membrane, as they were on the gel. The DNA is then fixed to the membrane by UV crosslinking (Stratagene Stratalinker, standard program) and the membrane is dried (for example in an oven at 75°C for 15 min). Next, the membrane is washed and treated with reagents that result in attachment of alkaline phosphatase to the DNA, according to the protocol recommended by New England Biolabs for chemiluminescence detection. This procedure takes ~45 min.
Finally, the membrane was washed briefly with ‘Triton buffer’, twice with ‘Tris–HCl, pH 9.5, solution’ for 5 min, submerged in a solution containing NBT/BCIP for ∼2 h, rinsed briefly with water and dried with a hair dryer, as recommended by Richterich and Church (7). Bands appear blue on a white background and remain visible for several months when the membrane is stored in the dark. The image on the membrane, processed as described above, may be scanned using a high resolution scanner, converting it to a digital form, and stored on a floppy disk. The image may be enlarged and viewed on a computer terminal screen or it may be printed on paper using an ordinary laser printer.

RESULTS AND DISCUSSION

Figure 4 shows results obtained with 35S-labeled DNA using X-ray film for detection. Duplicate loadings of the samples (GATCGATC) were performed using the central eight teeth of the loading device. Despite the small size of the gel (an effective length of ∼6.0 cm), single base resolution is apparent up to ∼150 nt from the primer (on the original autoradiograms). DNA fragments of lengths >150 nt from the 3′-end of the primer run in the upper third of this gel. Even on the prints the four consecutive C residues at nt 61–64 from the primer are easily resolved, as are the three consecutive T residues at ∼100 nt from the primer. Resolution compares favorably with that obtained using commercial sequencing gels. Moreover, the resolution of the longer fragments continues to improve as the gels are run for increasingly longer times, just as with ordinary gels (not shown).

Technical problems encountered were: occasional smearing, slanting, and band distortion. We feel that these relatively minor disturbances can be eliminated by taking care in making and setting up the gels and by thermostating. We have recently found that placing the gel on a 0.125 inch thick aluminum plate serves to eliminate smearing and that taking care that the wicks are properly cut to size and are placed straight serves to eliminate slanting. The maximum voltage that can be used has not been determined.

Using DNA samples labeled with 35S gave good results and the gels are easily and quickly prepared for film exposure. However, as with large gels, film exposure times of 16 h or longer are required in order to visualize the sequencing ladders. In order to be able to obtain results more quickly we tried non-radioactive detection using a biotinylated primer. The gel was easily and quickly blotted and the membrane processed as described in Materials and Methods. The gel-size membrane was incubated with 2 ml volumes of reagents in 12 ml tubes and washed with 25 ml volumes in 50 ml tubes. Thus much smaller quantities of (the fairly expensive) reagents are required to process the small membrane than the quantities needed to process a membrane the size of a traditional sequencing gel. We found that chemiluminescent detection produced somewhat ‘fuzzy’ bands. However, colorimetric detection (as described in Materials and Methods) produced bands that were nearly as sharp as those obtained using 35S. Figure 5 shows the image of a developed membrane scanned at 600 d.p.i., enlarged and printed out on paper using an ordinary laser printer. An 8-fold enlarged image of this gel could be read accurately up to ∼150 nt from the primer. Results can be obtained in ∼4 h from the time the gel was loaded.

The band resolution is certainly compromised in the colorimetric detection by substrate diffusion. It is also likely that band resolution is compromised by the granularity of the X-ray film for 35S-labeled samples. Thus detection of fluorescently labeled DNA directly in the gel might lead to improved performance. Development of a suitable fluorescence detection system should be possible because the sensitivity of detection of the 35S-labeled DNA and biotinylated DNA in the miniaturized system appears to be about the same as that for large gels, despite the much smaller sample volumes applied.

It should be possible to increase the number of samples that can be run per gel. This could be accomplished by reducing the width of the teeth on the sample loading device and also by reducing the tooth spacing. Also, it should be possible to use wider (50 mm) microscope slides, which are commercially available, to double the width of the gel. Perhaps as many as 40 samples/gel could be run. Additionally, sample loading as described here is amenable to automation. Placing small volumes of samples on the teeth of the sample loading device and then stamping them into the gel is a simpler operation for a robotic device than pipetting samples into the wells or slots of a vertical (urea-containing) gel.

A unique feature of this system is the very small sample volumes used. Approximately 50 nl deposited onto a tooth of the loading device is sufficient. When the emerging nanotechnologies make it possible to perform PCR and other reactions with sub-microliter volumes (8) the results described here indicate that it will be possible to obtain DNA sequence information from such samples.

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REFERENCES