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Genetics

Manifold sequencing: Efficient processing of large sets of sequencing reactions

(nucleotide sequence/solid support/manifold)

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ABSTRACT **Automated instruments for DNA sequencing greatly simplify data collection in the Sanger sequencing procedure. By contrast, the so-called front-end problems of preparing sequencing templates, performing sequencing reactions, and loading these on the instruments remain major obstacles to extensive sequencing projects. We describe here the use of a manifold support to prepare and perform sequencing reactions on large sets of templates in parallel, as well as to load the reaction products on a sequencing instrument. In this manner, all reaction steps are performed without pipetting the samples. The strategy is applied to sequencing PCR-amplified clones of the human mitochondrial D-loop and for detection of heterozygous positions in the human major histocompatibility complex class II gene *HLA-DQB*, amplified from genomic DNA samples. This technique will promote sequencing in a clinical context and could form the basis of more efficient genomic sequencing strategies.**

Large-scale genomic sequencing projects now under way are paralleled by an increasing interest to determine the nucleotide sequences of specific genes in patient samples. These efforts have prompted the development of a number of automated instruments to record nucleotide sequence data (1, 2). Other time-consuming aspects of collecting nucleotide sequence information, besides recording the information, include preparing the sequencing templates and generating and loading the sequencing reaction products on gels. Solid-phase sequencing of PCR products is an efficient method of preparing sequencing templates that results in high-quality sequence data (3, 4). We demonstrate in this paper a solid-phase-based method to process, in parallel, large sets of reactions up to and including the point where the sequencing reaction products are loaded on an automated sequencing instrument. In this strategy, sequencing templates are isolated and handled by using a solid support with 96 prongs projecting into individual reaction wells. Of crucial importance for our approach was the development of a very efficient means of immobilizing amplified sequencing templates on the limited surface available on the manifold support. This effect was achieved by creating a porous surface layer on the polystyrene device (5). In this manner, sufficient avidin can be coupled to individual prongs of the support in order to bind and sequentially transfer sets of biotin-labeled templates for fluorescence sequencing reactions through the series of reaction steps preceding gel analysis (Fig. 1).

MATERIALS AND METHODS

Solid Supports. Comb-shaped devices, similar to those used when casting sequencing gels to create slots for loading samples, were cut out of sheets of 100-μm biaxial polystyrene (Nordbergs Tekniska AB, Sundbyberg, Sweden) (Fig. 1*A*). As an alternative, a commercially available polystyrene device was used. This support has a set of 96 ball-and-stick extensions, projecting into individual wells of a microtiter plate located underneath (Falcon).

Avidin-coated supports were constructed essentially as previously reported (5). Briefly, 6 ml of Sepharose particles (HiTrap, *N*-hydroxysuccinimide-activated HP Sepharose, Pharmacia) were washed with three 10-ml portions of ice-cold 1 mM HCl on a sintered glass funnel. The particles were quickly washed in 1 M NaCl/0.4 M carbonate buffer, pH 8.3, and then incubated for 1 hr with 5 ml of the above buffer containing avidin at 2 mg/ml. The avidin-conjugated particles were blocked for 15 min in 0.1 M ethanolamine buffer at pH 8.3, washed in 0.1 M acetate buffer at pH 4.0 with 1 M NaCl, and used immediately or stored in 0.1 M Tris·HCl buffer, pH 7.3 with 0.02% (wt/vol) NaN_3. The Sepharose surface should not be allowed to dry at any time during this process.

Avidin-conjugated Sepharose particles were washed with distilled water, dried with three 5-ml portions of methanol, and then equilibrated in three 5-ml portions of triethylamine. The solid was transferred to a suitable vessel and triethylamine was added to obtain a slurry of about 50% (vol/vol) particles. The polystyrene supports were washed with ethanol for 20 min in an ultrasonic bath, and the particles were then grafted onto the tips of the projections by submerging these in the slurry for ≈2 sec, followed by evaporation of the triethylamine in air and repeating this process twice. Loosely bound particles were removed by rinsing the supports in distilled water. The manifolds were stored until use in 100 mM Tris·HCl, pH 7.5/0.02% NaN_3. Before use, the supports were washed in 1 M NaCl/100 mM Tris·HCl, pH 7.5/0.1% Triton X-100 (buffer A).

PCR Amplification. Biotinylated templates for sequencing the mitochondrial D-loop, cloned in plasmid pUC18, were prepared by amplification of the plasmid inserts. Single bacterial colonies were picked and suspended in 100 μl of water, and bacterial cells were lysed by heating at 100°C for 5 min. Aliquots (1 μl) of the supernatants were used as templates in 100-μl PCR mixtures containing 50 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/200 μM each deoxynucleoside triphosphate (dNTP) (PCR buffer), one 5′-biotin-labeled oligonucleotide (JS1B: 5′-biotin-GAGCGGATAACAATTTCACACAGG-3′) and one unmodified oligonucleotide (JS2: 5′-GCCAGGGTTTTCCCAGTCACGA-3′) at 0.1 μM each, and 5 units of *Taq* DNA polymerase. Primers JS1B and JS2 were designed to amplify sequencing templates cloned in pBluescript and pUC vectors. During PCR, the temperature cycle—94°C for 1 min, 55°C for 1 min, and 72°C for 2 min—was repeated 25 times.

PCR amplifications of exon 2 of the major histocompatibility complex class II gene *HLA-DQB* were performed in 100 μl of PCR buffer containing 0.5 μg of genomic DNA, 1 μM oligonucleotides DB130 (5′-AGGGATCCCCGCAGAGGATTTCGTGTACC-3′) and DB131 (5′-TCCTGCAGGGC-

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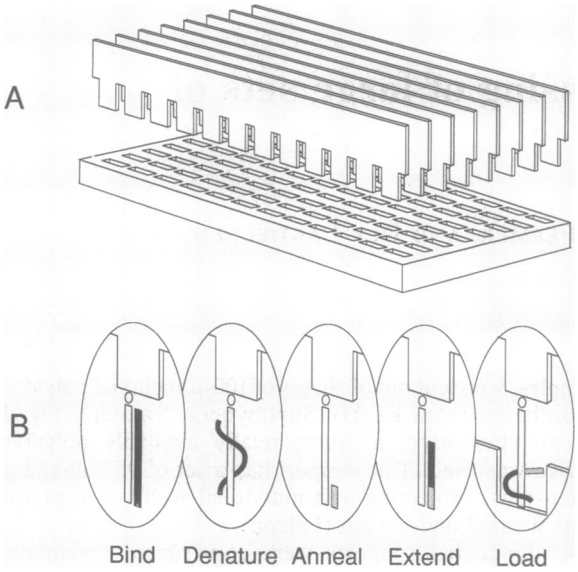

FIG. 1. Schematic description of the manifold support and the steps of the sequencing reactions. (*A*) A manifold device used for transferring sequencing reaction intermediates was constructed from polystyrene and modified with avidin-coupled Sepharose particles. (*B*) Biotin-labeled PCR products were used as templates for sequencing reactions by (*i*) binding the products to the support, (*ii*) removing the complementary strands by denaturation followed by washes, (*iii*) annealing the sequencing primer, (*iv*) performing the dideoxy-terminated extension reactions, and (*v*) transferring reaction products to the gel, using the manifold support.

GACGACGCTCACCTCCCC-3′) (6, 7), and 5 units of *Taq* polymerase. The temperature cycle of 94°C, 60°C, and 72°C for 1 min each was carried out 25 times. The amplified products were diluted 1:10,000 and subjected to a second PCR with nested primers. Reactions were performed in 100 μl of PCR buffer containing 0.1 μM biotinylated oligonucleotide BGH29 (5′-biotin-GAGCTGCAGGTAGTTGTGTCTGCACAC-3′), and 1 μM oligonucleotide GH28U (5′-TGTAAAACGACG-GCCAGTCTCGGATCCGCATGTGCTACTT-3′), and 5 units of *Taq* polymerase. The oligonucleotide GH28U includes the sequence of the M13 universal sequencing primer at the 5′ end, in order to allow annealing of a standard sequencing primer to the PCR products. The samples were amplified in 28 temperature cycles of 94°C, 60°C, and 72°C for 1 min each.

Biotinylated PCR products, used to assay the binding capacity of the supports, were amplified from several cloned templates. Reactions were performed as described for the mitochondrial D-loop. The amplification products were radiolabeled during amplification by including 0.01 volume of [α-^{32}P]dCTP (10 mCi/ml, 3000 Ci/mmol, DuPont; 1 Ci = 37 GBq) in the PCR mixtures. Unincorporated radioactive nucleotides were removed with a Sephadex G-50 spin column (6). The molar amount of radiolabeled material was estimated by comparison to a molecular size standard in an agarose gel.

Binding of Sequencing Templates to the Support. After temperature cycling, 20-μl aliquots of each amplification reaction mixture were distributed in four wells of reaction plates, and 5 μl of 5 M NaCl was added. The reaction plates were made by drilling wells, 1.5 mm × 5.5 mm and 5 mm deep, out of blocks of Delrin (Fig. 1*A*). The commercially available ball-and-stick supports were incubated in wells of a microtiter plate (Techne Laboratories, Princeton, NJ) with the addition of 20 μl of buffer A per well. Biotinylated PCR products were immobilized on the avidin-coated supports in a 2-hr incubation at 42°C in a humidified atmosphere.

Sequencing Reactions. Solid supports with immobilized PCR products were incubated in 0.1 M NaOH/1 M NaCl/0.1% Triton X-100 for 15 min on a shaking platform to release the complementary DNA strands. The supports were then washed once in the same solution, once in buffer A, and once in 10 mM Tris·HCl, pH 7.5/50 mM NaCl. After washing, the supports were placed in sets of wells containing 0.5 pmol of a fluorescein-labeled sequencing primer (5′-fluorescein-CGACGTTGTAAAACGACGGCCAGT-3′; M13 universal primer; Pharmacia) in 25 μl of 10 mM Tris·HCl, pH 7.5/50 mM NaCl. The reaction mixtures were heated at 65°C for 10 min and allowed to cool to room temperature. Next, the supports were incubated for 15 min at 42°C in reaction wells containing 20 μl of 40 mM Tris·HCl, pH 7.5/11 mM dithiothreitol/4 mM $MnCl_2$/29 mM isocitrate/23 mM NaCl/0.46 mM each dNTP/2.3 μM one dideoxynucleoside triphosphate (ddNTP) and 4 units of T7 DNA polymerase (Pharmacia) (7). Reaction products immobilized on the comb-shaped support were loaded on sequencing gels as described below. Alternatively, 5 μl of 0.1 M EDTA was added to the wells, and the reaction products were stored at −20°C. Reactions performed with the ball-and-stick supports were stopped by immersing the supports in wells containing 20 μl of formamide. After 10 min at room temperature, the reaction mixtures were heated at 90°C for 2 min, and the supports were removed. Ten microliters of each reaction mixture was then loaded onto a fluorescence sequencing instrument by pipetting, or the reaction mixtures were stored at −20°C.

Loading of Sequencing Reactions Products with a Solid Support. The comb-shaped supports were employed to add the reaction products directly to the gel. First, the sequencing gel was heated to 50°C and formamide was distributed with a syringe across all of the wells under running buffer. Next, the supports were immersed in the wells for 10 min and then removed before electrophoresis was begun. During electrophoresis, the temperature of the gel was lowered to 40°C.

RESULTS

Binding of PCR Products. Large sets of sequencing reactions may be handled in parallel by using a manifold support. Moreover, the same set of supports can be used to serially transfer the reaction intermediates throughout all the steps from the preparation of the template up to the point when the reaction products are loaded on a sequencing gel (Fig. 1). We first examined the capacity of the manifold supports to bind biotinylated PCR products of different sizes. Fig. 2 illustrates that 2.5 pmol of a 280-base-pair product can be immobilized on each tooth of the manifold. Approximately 0.5 pmol of an

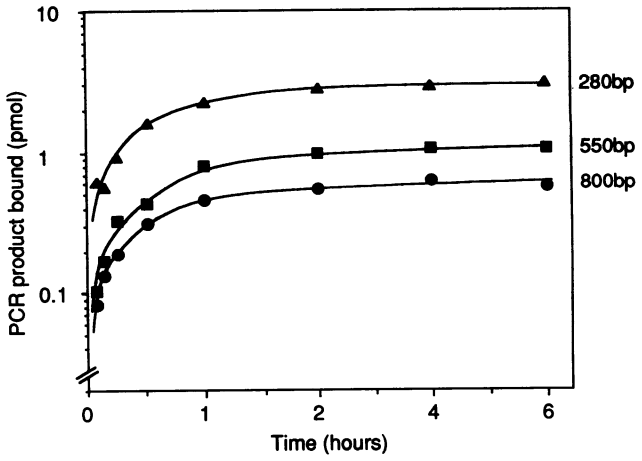

FIG. 2. Kinetics of binding to the manifold supports for PCR products of the indicated sizes. PCR aliquots (20 μl) were incubated with the prongs of the supports for various times at 42°C. Binding of radiolabeled amplification products was analyzed by Cerenkov counting.

800-base-pair PCR product, present in a 20-μl PCR mixture, was bound to the support in a 2-hr incubation. The amount of single-stranded template required for a fluorescence sequencing reaction is of the order of 0.2 pmol (data not shown).

Manifold Sequencing. The manifold sequencing strategy was applied to analyze human mitochondrial D-loop sequences cloned in a plasmid vector. Analysis of this highly polymorphic sequence is valuable in studies of the degree of relationship among human populations and in forensic applications (8).

Sets of up to 24 different biotinylated PCR products, amplified from recombinant bacterial clones, were immobilized on four different avidin-coated prongs each and transferred to other wells for denaturation. The supports with single-stranded template molecules were then introduced into reaction wells containing fluorescence-labeled sequencing primers. After annealing, the supports were moved to a new set of wells for the sequencing reactions. The reaction products were eluted in formamide and the samples were loaded onto a sequencing gel by pipetting. An example of a sequencing reaction analyzed on an automated sequencing instrument (ALF automated laser fluorescence DNA sequencer, Pharmacia) is shown in Fig. 3. The last 180 bases of the fragment are shown, ending with 50 bases of vector sequence. The results demonstrate that a high-quality sequence chromatogram was obtained, with a high signal-to-noise ratio. The sequence is equivalent in quality to that obtained by using highly purified M13 single-stranded templates. It is readable throughout the 450-base-pair PCR product with no need for manual corrections after the computer analysis.

Gel Loading of Sequencing Reactions Using the Manifold Support. One of the hurdles of extensive sequencing is the loading of reaction products onto sequencing gels. This step introduces a risk of mix-ups and requires tedious, precise work. Specialized automated instruments have been constructed to overcome this difficulty. We reasoned that a manifold support could also serve to load the sequencing reaction products on the gel. The commercially available manifold support cannot be used for this purpose, due to the shape and spacing of the prongs, but the supports cut out of polystyrene sheets are designed to fit into the slots of a sequencing gel. In order to investigate the solid-support-loading procedure, extension reactions from support-bound templates were generated with ddTTP as a terminator and eluted directly in the wells of a sequencing gel. The comb support was removed before electrophoresis was begun, in order to avoid distortion in the electric field during the run. For comparison, identical dTTP reactions were removed from the supports by conventional denaturation in separate wells, and loaded onto the gel by pipetting. The two techniques of adding reaction products to the gel are compared in Fig. 4, where two ddTTP-terminated extension reactions, loaded onto a fluorescence-sequencing gel, are shown superimposed. It is evident that sequencing reaction products were efficiently removed from the solid support in the gel. Furthermore, there was no indication of reduced resolution or distortion of the migration pattern when the comb-shaped supports were used for adding the reaction products to the gel.

Sequencing Heterozygous *DQB* Genes with Direct Loading. In a clinical context, sequence analysis of patient samples

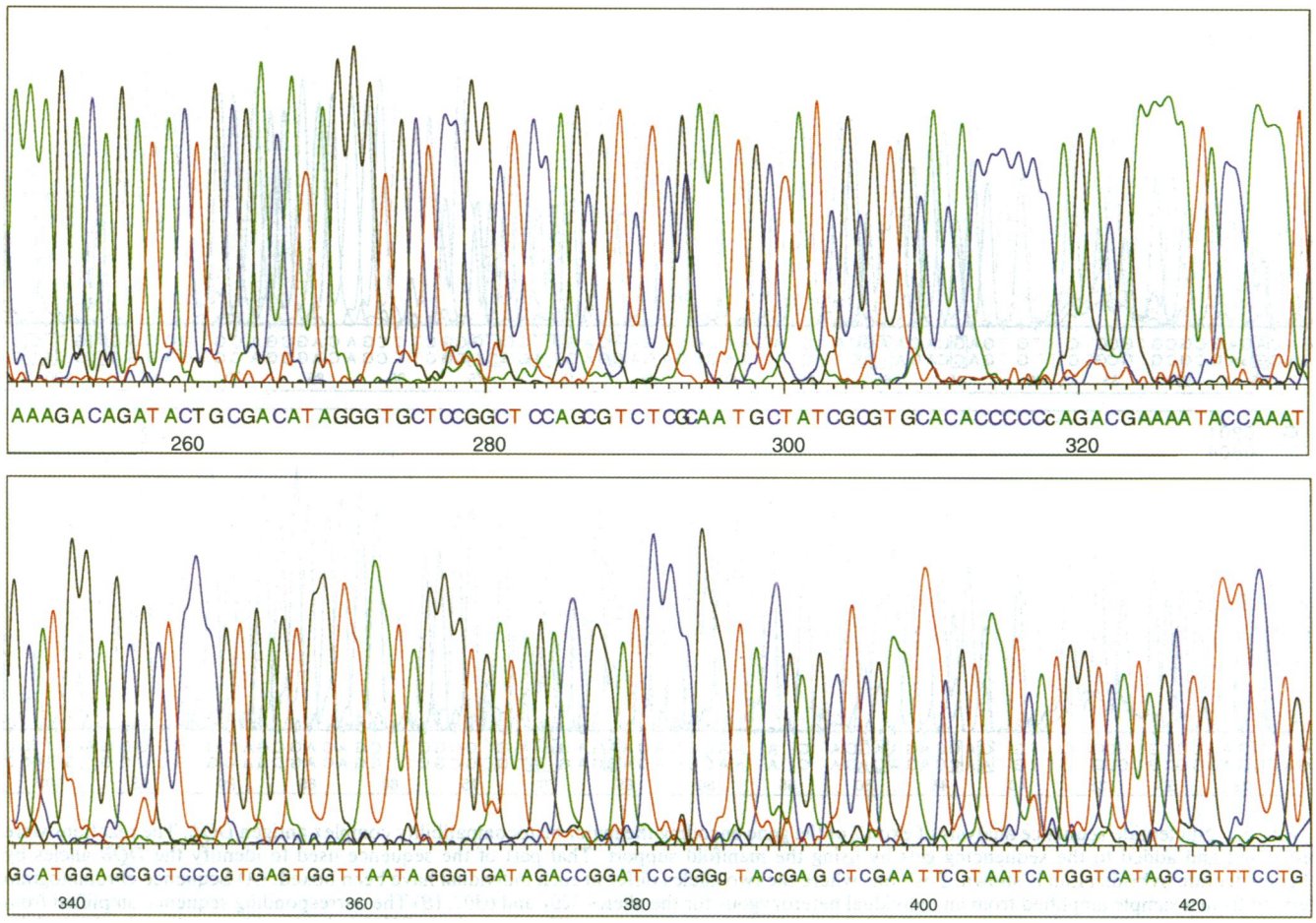

FIG. 3. Chromatogram of a human mitochondrial D-loop segment, sequenced by using the manifold support. The last 180 nucleotides of a cloned and amplified 450-base-pair product are shown.

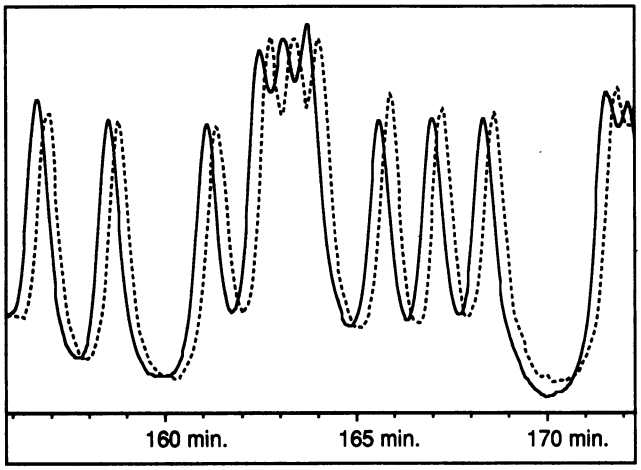

FIG. 4. Comparison between extension reaction products generated in the presence of a chain-terminating dTTP-analog, ddTTP, and using a manifold support. The reaction products were either denatured separately and then added to the sequencing gel by pipetting (- - - - -) or the extension products were released from the support directly in the sequencing gel (———).

frequently requires that heterozygous nucleotide positions can be identified. Such heterozygous positions correspond to mutations or polymorphisms distinguishing the two chromosome homologs. We investigated the potential of the manifold sequencing strategy in a routine sequencing application by analyzing PCR products derived from a highly polymorphic segment of the major histocompatibility complex class II gene *DQB*. This region is of interest for genotyping individuals in forensics and in connection with tissue transplantation (9, 10). Exon 2 of the *DQB* gene was amplified by PCR and sequenced. The reaction products were loaded on the gel by immersing the supports in the wells of a sequencing gel. Fig. 5 demonstrates the results from an analysis of two individuals previously shown to be heterozygous for the *DQB* gene by genotyping with sequence-specific oligonucleotide probes. The quality of the sequence data permits the identification of heterozygous nucleotide positions. These positions correspond to known differences among the alleles (11). In this manner, complex genotypes may be defined in a simple procedure.

DISCUSSION

Several techniques have been proposed to replace the Sanger method of generating and analyzing nested sets of fragments in order to reveal the sequence of nucleotides along a template strand. Such techniques include hybridization-based analyses (12–14), monitoring the stepwise incorporation of individual nucleotides on sets of templates (15), and examination of individual DNA molecules, either by scanning-probe microscopy (16) or by identifying individual nucleotides sequentially cleaved from one end of a molecule by an exonuclease (17).

Despite these efforts, progress in sequencing methodology has primarily been represented by incremental gains in efficiency of the standard Sanger procedure. A series of auto-

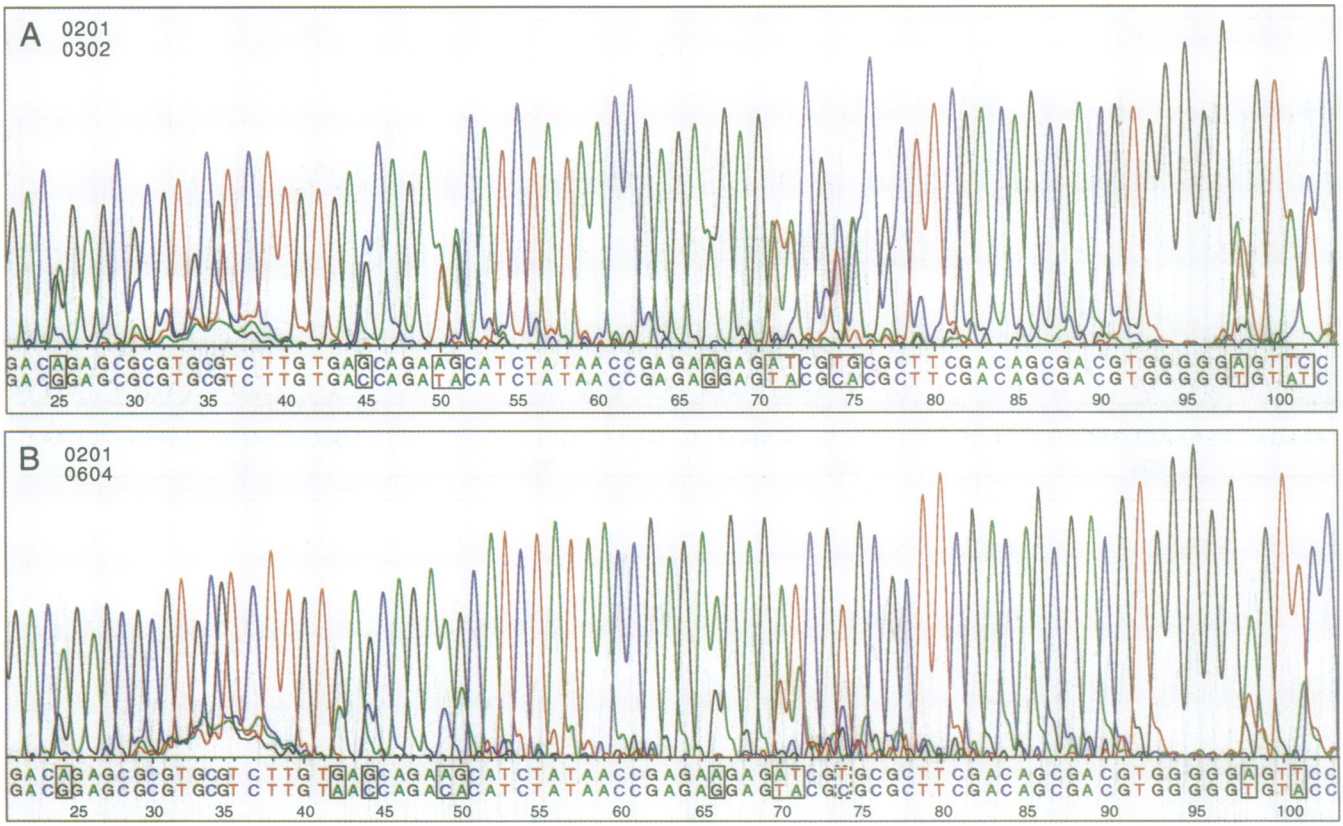

FIG. 5. Nucleotide sequence analysis of exon 2 of the gene encoding the major histocompatibility complex antigen DQβ. The reactions were processed and added to the sequencing gels by using the manifold support. That part of the sequence used to identify the *DQB* alleles by oligonucleotide hybridization is shown. Positions where the two alleles differ in each individual have been boxed. (*A*) Sequence chromatogram derived from a sample amplified from an individual heterozygous for the alleles *0201* and *0302*. (*B*) The corresponding sequence amplified from an individual heterozygous for the alleles *0201* and *0604*. In this sample, the amplification primer GH29 hybridized less well to the *0604* allele, resulting in a weaker representation of this allele in the chromatogram. Despite this, the instrument correctly identified each of the sequence discrepancies except nucleotide position 74 (dashed box).

mated, fluorescence-based sequencers are now available (18–21). In this paper we establish a strategy that permits processing of Sanger sequencing reaction through the steps preceding gel separation and data recording with the aid of a manifold device. This technique has several attractive features: up to 96 or even greater numbers of sequencing reactions can be performed in parallel with little increase in effort over the handling of a single one; the samples are serially transferred through all processing steps, including the loading of samples onto the gel; and the risk of sample contamination or mix-up is minimized. The technique may be used for radioactive and fluorescence sequencing alike. A related manifold approach has previously been presented for the Maxam–Gilbert sequencing method (22). To reach the required binding capacity, we have developed an effective way of increasing the surface available for coupling avidin onto multipronged supports, by attaching porous particles to the plastic surface in a simple step (5). This surface modification procedure is applicable to a wide range of combinations of molecules, particles, and macroscopic supports.

Because of the properties mentioned above, the strategy presented here is well suited for the wide-scale application of sequencing as a clinical tool. The standardized format ensures that optimal, reproducible reaction conditions can be defined and then achieved consistently. Moreover, the reagents could be prepared in a stabilized form distributed in the reaction vessels (23), further reducing the potential for operator error. A similar assay format can be applied in other genetic tests, also performed by using a manifold support, but loaded and read on other instruments (24).

The use of a manifold support will also benefit fully-automated sequencing procedures, since simultaneous processing of large sets of reactions can increase throughput. In addition, the transport of individual reactions with a manifold rather than by liquid-handling steps represents a technological simplification. The present sequencing strategy is particularly suitable for amplified samples, whether derived from genomic DNA or from cloned DNA, but efforts to bind substantially longer templates may open possibilities for efficient "genomic walking" sequencing approaches.

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1. Trainor, G. L. (1990) *Anal. Chem.* **62,** 418–426.
2. Hunkapiller, T., Kaiser, R. J., Koop, B. F. & Hood, L. (1991) *Science* **254,** 59–67.
3. Hultman, T., Ståhl, S., Hornes, E. & Uhlén, M. (1989) *Nucleic Acids Res.* **17,** 4937–4946.
4. Hopgood, R., Sullivan, K. M. & Gill, P. (1992) *BioTechniques* **13,** 82–92.
5. Parik, J., Kwiatkowski, M., Lagerkvist, A., Samiotaki, M., Lagerström, M., Stewart, J., Glad, G., Mendel-Hartvik, M. & Landegren, U. (1993) *Anal. Biochem.* **211,** 144–150.
6. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
7. Voss, H., Schwager, C., Kristensen, T., Duthie, S., Olsson, A., Erfle, H., Stegemann, J., Zimmermann, J. & Ansorge, W. (1989) *Methods Mol. Cell. Biol.* **1,** 155–159.
8. Vigilant, L., Pennington, R., Harpending, H., Kocher, T. D. & Wilson, A. C. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9350–9354.
9. Bugawan, T. L. & Erlich, H. A. (1991) *Immunogenetics* **33,** 163–170.
10. Allen, M., Saldeen, T., Pettersson, U. & Gyllensten, U. (1993) *J. Forensic Sci.* **38,** 554–570.
11. Marsh, S. G. E. & Bodmer, J. G. (1992) *Tissue Antigens* **40,** 229–243.
12. Drmanac, R., Labat, I., Brukner, I. & Crkvenjakov, R. (1989) *Genomics* **4,** 114–128.
13. Khrapko, K. R., Lysov, Y. P., Khorlyn, A. A., Shick, V. V., Florentiev, V. L. & Mirzabekov, A. D. (1989) *FEBS Lett.* **256,** 118–122.
14. Southern, E. M., Maskos, U. & Elder, K. J. (1992) *Genomics* **13,** 1008–1017.
15. Rosenthal, A. & Brenner, S. (1993) *Genome Mapping and Sequencing* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 222.
16. Lindsay, S. M. & Philipp, M. (1991) *Genet. Anal. Tech. Appl.* **8,** 8–13.
17. Davis, L. M., Fairfield, F. R., Harger, C. A., Jett, J. H., Keller, R. A., Hahn, J. H., Krakowski, L. A., Marrone, B. A., Martin, J. C., Nutter, H. L., Ratliff, R. L., Shera, E. B., Simpson, D. J. & Soper, S. A. (1991) *GATA* **8,** 1–7.
18. Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Conell, C. R., Heiner, C., Kent, S. B. H. & Hood, L. E. (1986) *Nature (London)* **321,** 674–679.
19. Ansorge, W., Sproat, B., Stegemann, J. & Schwager, C. (1986) *J. Biochem. Biophys. Methods* **13,** 315–323.
20. Prober, J. M., Trainor, G. L., Dam, R. J., Hobbs, F. W., Robertson, C. W., Zagursky, R. J., Cocuzza, A. J., Jensen, M. A. & Baumeister, K. (1987) *Science* **238,** 336–341.
21. Middendorf, L. R., Bruce, J. C., Bruce, R. C., Eckles, R. D., Grone, D. L., Roemer, S. C., Sloniker, G. D., Steffens, D. L., Sutter, S. L., Brumbaugh, J. A. & Patonay, G. (1992) *Electrophoresis* **13,** 487–494.
22. Rosenthal, A., Jung, R. & Hunger, H.-D. (1987) *Methods Enzymol.* **155,** 301–331.
23. Ortlepp, S. A. & McKay, I. A. (1989) *BioTechniques* **7,** 1110–1115.
24. Samiotaki, M., Kwiatkowski, M., Parik, J. & Landegren, U. (1994) *Genomics*, in press.