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Real-time detection of allele-specific polymerase chain reaction products by automated ultra-thin-layer agarose gel electrophoresis

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Abstract

Ultra-thin-layer agarose gel electrophoresis, a novel combination of agarose slab gel electrophoresis and capillary gel electrophoresis was introduced in conjunction with laser-induced fluorescence (LIF) scanning detection for the analysis of polymerase chain reaction (PCR) products. Allele-specific fragments, amplified from genomic DNA of patients with congenital adrenal hyperplasia (most often caused by mutations of 21-hydroxylase gene, CYP-21), were used as a model system to investigate the applicability, sensitivity and resolving power of the method. The allele-specific products were generated by PCR and separated by ultra-thin-layer agarose gel electrophoresis. The double-stranded DNA fragments were easily visualized in real-time via complexation during the separation process by the intercalator dye TO-PRO-3 which was part of the separation gel–buffer system. In this way, the migrating dsDNA–dye complexes were detected in real-time by a scanning LIF detection system with sub-nanogram sensitivity. The system employs a 632-nm solid-state laser and an avalanche photodiode detector scanning to the separation platform by means of a fiber bundle system. Automated ultra-thin-layer agarose gel electrophoresis with 'on the fly' TO-PRO-3 staining of dsDNA fragments and LIF detection system proved to be a very fast, high-throughput separation method for individual or multiplexed PCR products, with excellent sensitivity.

Keywords: Polymerase chain reaction; Ultra-thin-layer agarose gel electrophoresis; DNA

1. Introduction

Agarose gel electrophoresis is one of the most frequently used separation techniques for the analysis of double-stranded (ds)DNA molecules [1]; in spite of this methodology it is still based on mostly manual processing. The technique requires post-separation evaluation of the separated bands usually by Coomassie Brilliant Blue, silver, ethidium bromide [2] and most recently by novel, high-sensitivity fluorescent-staining procedures [3]. In case of ethidium bromide staining, the dye can be part of the separation medium, which also enhances separation selectivity [4]. The thickness of the commonly used agarose gels ranges between 1 and 5 mm. During electrophoretic separation, agarose serves a dual purpose: as anti-convective medium and as sieving material. Recently, capillary electrophoresis has emerged as a novel, high-performance DNA analysis tool, due to its inherent rapid and effective separations, as well as high detection sensitivity by laser-induced fluorescent detection [5]. Applications have been shown employing intercalator dyes, such as ethidium bromide and thiazole orange [6], to accommodate the commonly used 488-nm argon-ion lasers and just recently TO-PRO-3 intercalator dye in

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conjunction with the 640-nm solid-state red diode laser [7]. The combination of these two well-established methodologies take advantage of the multilane separation format of slab gel electrophoresis and the high separation efficiency of capillary electrophoresis.

Detection of DNA point mutations is a widely used diagnostic tool, currently implemented by methods such as solid-phase micro-sequencing, single-stranded conformation polymorphism, direct sequencing of polymerase chain reaction (PCR) products, allele-specific oligo-hybridization, etc. [8]. Allele-specific amplification (ASA) is a very simple and highly reliable technique to identify point mutations [9]. In principle, ASA can detect any previously identified point mutation sites by employing a specifically designed primer, which anneals with its 3′ end right at the site of mutation of interest. Using Taq DNA polymerase, without any 3′ exonuclease activity, amplification of this allele-specific fragment will start only if the 3′ end of the primer matches with the mutation present in the DNA investigated. The resulting PCR fragment is then identified by means of agarose gel electrophoresis as a proof of the mutation present in the gene examined. In the case where there is no mutation at that particular site of interest, due to the mismatch at the 3′ end of the primer, the Taq polymerase does not replicate DNA, therefore no fragment is formed. An internal control amplification is always suggested, providing a positive control for the PCR reaction.

Congenital adrenal hyperplasia (CAH) is often caused by single-point mutations in the 21-hydroxylase gene [10]. Wedell and Luthman [11] developed a PCR-based ASA method for detection of various point mutations on the 21-hydroxylase gene (CYP-21), providing a suitable PCR-based system for clinical screening of mutations without any interference with the pseudogene (CYP-21P). In the present study, we used their model system to produce allele-specific DNA fragments of medical importance in order to evaluate the sensitivity and resolving power of the automated ultra-thin-layer agarose gel electrophoresis system with LIF detection. The allele-specific PCR products were labeled by the intercalator dye TO-PRO-3 during the electrophoresis separation and the dsDNA–dye complexes were visualized in real-time by a scanning laser-induced fluorescence system, employing a 632-nm solid-state laser and avalanche photodiode detector.

In this paper we present a novel separation technique of automated, ultra-thin-layer agarose gel electrophoresis, that combines the advantages of agarose slab gel electrophoresis with the dimensions of capillary gel electrophoresis to obtain rapid and efficient separation of dsDNA molecules in a multilane format separation platform.

2. Materials and methods

2.1. Chemicals

Tris, boric acid and EDTA were obtained from ICN (Costa Mesa, CA, USA), all in electrophoresis grade. In all the experiments, 2% Super Resolution Agarose (McFrugal, Del Mar, CA, USA) was used in 50 m*M* Tris, 50 m*M* boric acid 2 m*M* EBTA (TBE) buffer (pH 8.3). The allele-specific primers [11] have been synthetized by the Agricultural Biology Center (Gödöllö, Hungary). DNA samples of patients with congenital adrenal hyperplasia were the kind gift of Dr. J. Sólyom (2nd Department of Pediatrics, Semmelweis University of Medicine School, Budapest, Hungary). The Taq DNA polymerase, the ΦX174 *Hae*III restriction digest fragment mixture and the Cy5-labeled 50-base pair (bp) ladder were from Pharmacia Biotech ALFexpress Sizer 50-500 (Uppsala, Sweden). The 100-bp dsDNA ladder was from McFrugal. The TO-PRO-3 intercalator dye was from Molecular Probes (Eugene, OR, USA).

2.2. Allele-specific PCR

Conditions of the allele-specific PCR were similar to that explained in Ref [11]. In the initial PCR the phenol-extracted human genomic DNA [12] was amplified to get a 2063-bp fragment with PCR primers carefully designed in a way to avoid amplification of the pseudogene. This 2063-bp fragment was then used as a template for the re-PCR by allele-specific primers. An internal control amplification (1263-bp fragment) was included in every PCR. A typical PCR reaction mixture contained: 10 m*M*

Tris–HCl (pH 9.0), 1.5 m*M* $MgCl_2$, 50 m*M* KCl buffer, 5% glycerol, 200 μ*M* of each dNTP, 0.5 μ*M* allele-specific primers, 0.2 μ*M* limiting primer, 1 U Taq DNA polymerase and 0.1 ng of target DNA or 1 μl of the first PCR product in a final volume of 20 μl. Thirty cycles at 96°C for 1 min, 54°C for 30 s and 72°C for 3 min were performed in an Ericomp (San Diego, CA, USA) thermal cycler.

2.3. Instrumentation

The G-6000 high-performance DNA analyzer (Genetic BioSystems, San Diego, CA, USA) was used in all the experiments with reversed polarity separation mode (anode at the detection side). The system employs a 640-nm diode laser for excitation and an avalanche photodiode for detection. A translation stage scans across the multilane separation platform collecting the emitted fluorescent signal [13] and the data are acquired and evaluated by a personal computer (Fig. 1). The separation platform was a 18×7.5-cm float glass cartridge with a 0.19-mm gap between the upper and lower glass plates, and two 15-ml buffer reservoirs at both ends. The super resolution agarose powder was suspended in 50 m*M* TBE buffer, boiled repetitively in a microwave oven until clearness, and was kept at 60°C until use. Two n*M* TO-PRO-3 intercalator dye was added to the melted agarose solution just before it was pumped into the separation platform. After 15 min of cooling/solidification, the gel-filled cartridge was ready to be used. The injection of the dsDNA standards and PCR products onto the ultra-thin-layer agarose gel was accomplished by membrane-mediated loading technology [14]. Small amounts of samples (0.5 μl) were spotted onto the tips of a 24-tab membrane loader (Genetic BioSystems) and carefully inserted into the separation platform. By applying the electric field, the samples were quantitatively loaded into the separation matrix from the membrane. The applied voltage was 750 V for all the experiments resulted in a 5–7-mA current during the separations. Aluminum heat-sinks were employed to hold the separation cartridge and to dissipate excess Joule heat (Fig. 2). The heat sinks were cooled to

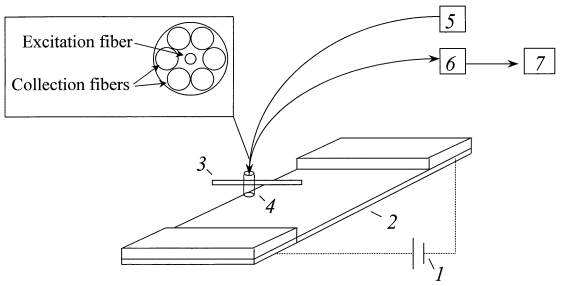

Fig. 1. Block diagram of the automated ultra-thin-layer agarose gel electrophoresis system with scanning laser-induced fluorescent detection. (1) High-voltage power supply; (2) ultra-thin-layer separation platform with built-in buffer reservoirs; (3) translation stage; (4) scanning lens set with fiber optic bundle; (5) 640-nm laser diode; (6) avalanche photodiode; (7) computer. Inset: fiber bundle with one central illumination and six surrounding collecting fibers.

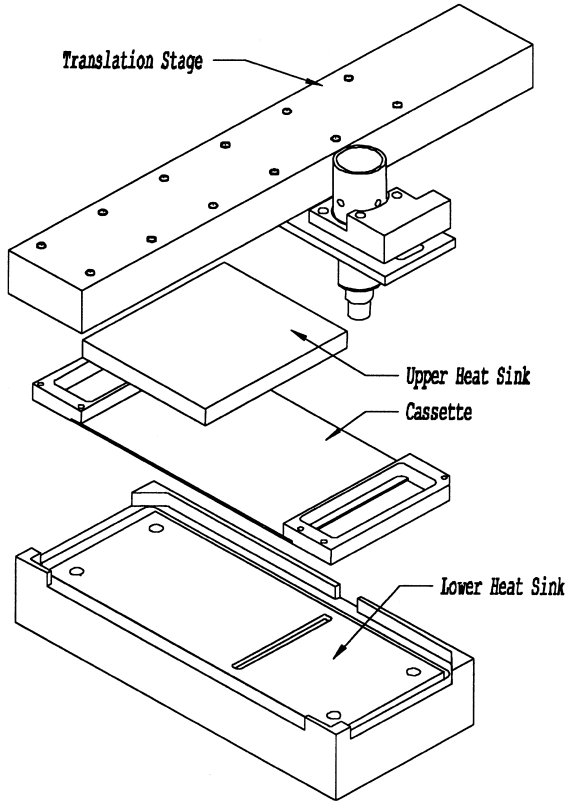

Fig. 2. Heat sink design for ultra-thin-layer agarose gel electrophoresis apparatus.

ambient temperature by a fan during separation. The agarose gel was replaced in the separation cartridge after each run, by simply melting the used gel in the cassette by a microwave oven and pumping in fresh melted one.

3. Results and discussion

3.1. Scanning detection system

Fig. 1 depicts the block diagram of the automated ultra-thin-layer agarose gel electrophoresis system used for the separation and detection of dsDNA fragments, including the high voltage electrophoresis power supply (1); the separation platform with built-in buffer reservoirs (2); the translation stage (3); the scanning lens set connected to the fiber optic bundle (4); the laser diode (5); the avalanche photodiode (6); and the computer (7) [13]. As the fluorescently labeled dsDNA molecules migrate through the detection area in the multilane separation platform, the lens set scans across the gel by means of the translation stage. The lens set is connected to the fiber bundle. The laser beam is focused into the central fiber of the bundle for excitation, and the surrounding collection fibers collect the fluorescent light (see inset in Fig. 2) emitted by the labeled sample components. The solid-state laser diode used in the system provided the 640-nm excitation beam. The emitted light was collected by an avalanche photodiode using a 682-nm wide band interference filter. This excitation/emission wavelength combination allowed the application of two fundamentally different fluorescent dsDNA labeling methodologies: covalent fluorophore tagging by the cyanine dye Cy5 and intercalation labeling by TO-PRO-3. In the first case, the DNA is covalently labeled via the use of Cy5-labeled primers, having 650 nm excitation and 659 nm emission maximums. In the second instance, dsDNA/TO-PRO-3 complex was used that has a published excitation maximum at 642 nm while the excitation maximum of the free un-complexed dye was found to be 631 nm [7]. The shift between the excitation maximums provided the possibility to obtain good detection limit for the dsDNA–dye complexes, even when extra intercalator dye was present in the separation medium. This later method allows easy labeling of dsDNA molecules during the electrophoresis step.

3.2. Real-time detection of dsDNA fragments

First, a mixture of Cy5-labeled dsDNA fragments (50–500 bp) was injected, electrophoresed and detected in order to show the separation power and the real-time detection capability of the system. Fig. 3 shows the separation of the 50-bp ladder on a 2% gel. With the effective separation distance of 6 cm, rapid ($<$15 min) and good resolution ($R_{s,average}>2.2$) of the individual fragments were obtained. The detection was accomplished in real-time by continuous scanning of the detection regime of the multilane separation platform during electrophoresis. Please note that detection of the migrating dsDNA molecules was accomplished in a timely manner in contrast to the traditional spatial agarose gel separations, assuring that all dsDNA molecules in the sample mixture travel the same distance from the injection point to the detection point (6 cm in this case). Similar to capillary gel electrophoresis, the larger, thus lower mobility fragments, will reach the detection regime later than the smaller ones; therefore, these larger fragments travel through a significantly longer distance to get detected compared to regular slab gel techniques [1,2]. This real-time band detection allows evaluation of the resulting separation directly on the computer screen. In Fig. 3, the line-like series of sharp bands after the first slightly fuzzy row of bands migrating around 9.5 min correspond to the Cy5-labeled primer front and partially interfere with the 50-bp fragment bands. Otherwise, all the double-stranded DNA fragments from 100 to 500 bp are well resolved (in 50-bp increments), and the entire separation took less than 14 min. By loading samples onto all 24 lanes, the high-throughput separation capability of the system was also demonstrated. As a comparison, this throughput corresponds to a 37-s separation of the same sample mixture by a single-lane device, such as capillary electrophoresis. The ultra-thin-layer format of the separation platform provides good heat dissipation during the electrophoresis, resulting in high separation efficiency and good resolution. Each band in Fig. 3 corresponds to 1 fmol dsDNA.

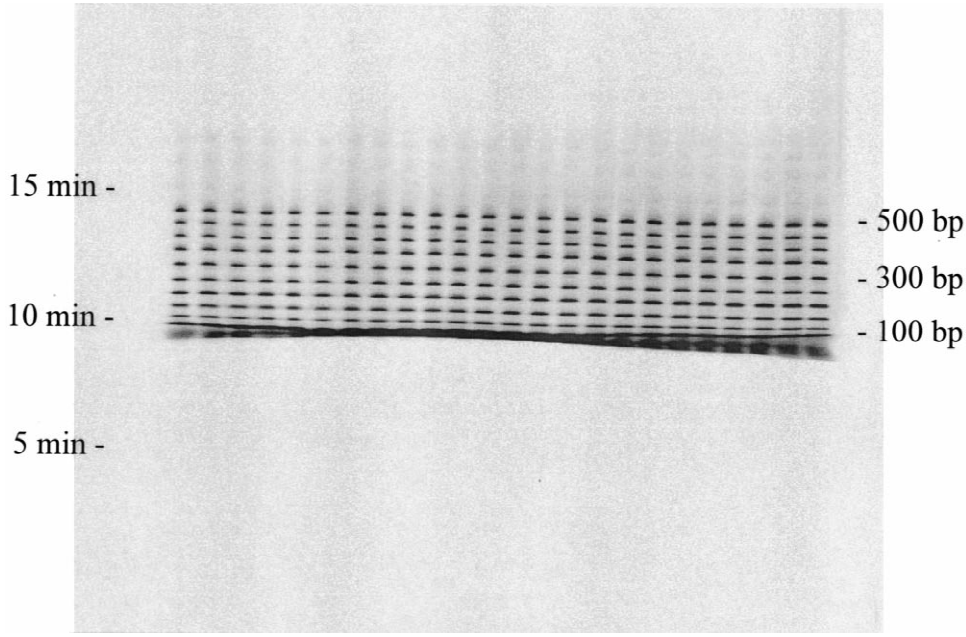

Fig. 3. Automated ultra-thin-layer agarose gel electrophoresis separation of Cy5-labeled 50-bp dsDNA ladder. Conditions: effective separation length, 6 cm; separation gel, 2% agarose in 50 m*M* TBE buffer; running buffer, 50 m*M* TBE (pH 8.3); applied voltage, 750 V; temperature, 24°C. Injection: 1 fmol/fragment per lane, mediated by a 24-tab membrane loader.

Detection and separation of non-prelabeled dsDNA molecules such as the mixture of ΦX174 *Hae*III digest restriction fragments can be accomplished by simply adding the intercalator dye TO-PRO-3 to the separation gel–buffer system (Fig. 4) in the appropriate concentration. In this experiment, 5 ng total dsDNA was loaded onto each tab of the membrane loader (e.g., this corresponds to 0.5 fmol of the 1353-mer). The loaded membrane was then placed into the separation cartridge and the dsDNA sample molecules were injected into the separation gel by the application of the electric field. As one can observe nice separation of the fragments was obtained. The particular gel and TO-PRO-3 concentration used in this experiment (2% and 2 n*M*, respectively) provided excellent separation of all fragments in less than 25 min in the chain length range of 72–1353 bp, except for the 271- and 281-bp fragments which were not fully resolved. The higher fluorescent signal of the longer fragments is due to the complexation characteristics, as longer fragments bind more dye [4]. Please note, that even the smallest (72 bp) fragment in the test mixture was well detected using LIF detection with 'on the fly' TO-PRO-3 intercalation staining.

3.3. Analysis of allele-specific PCR products

Fig. 5 illustrates the applicability of ultra-thin-layer agarose gel electrophoresis for the analysis of allele-specific PCR products, obtained from samples of patients with congenital adrenal hyperplasia. Parallel to the PCR samples, a 100-bp ladder was also injected and separated for fragment size evaluation purpose. Ten-fold diluted PCR reaction mixture was directly applied (0.5 μl) onto the tabs of the membrane loader, without any sample purification. The DNA sample of Patient-1 exhibited a 322-bp allele-specific fragment (migration time, 11 min) suggesting a mutation at position 1004 on the 21-hydroxylase gene (Table 1, first row). In this instance, the high-molecular weight fragment appearing above the position of the control fragment (migration time, 21 min) derived from the first PCR reaction. In the DNA sample of Patient-2, formation of the 705-bp allele-specific fragment (migration

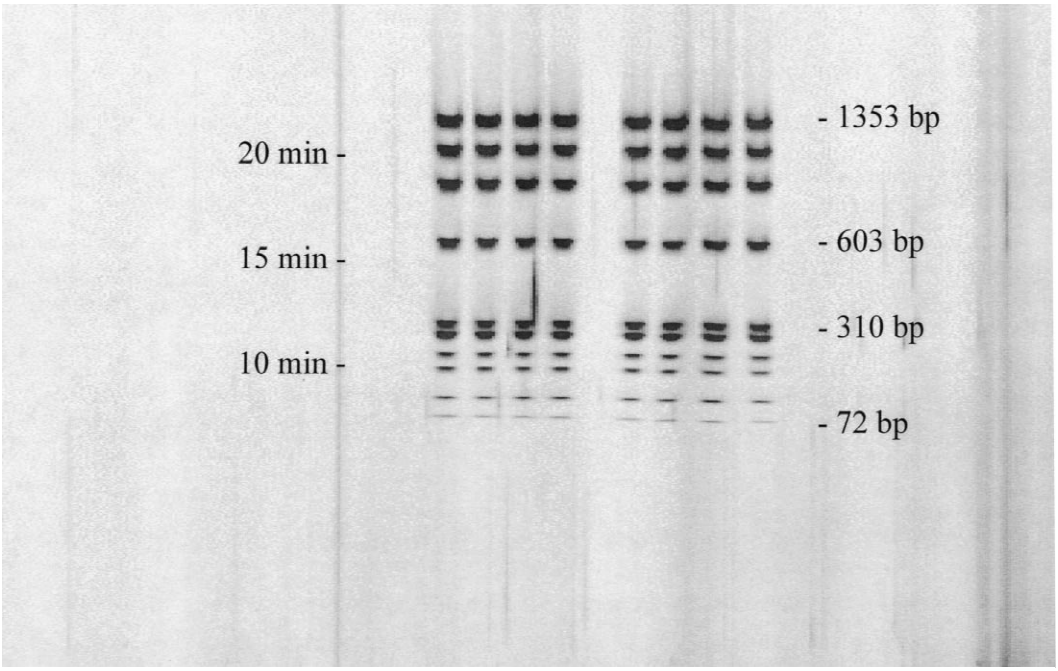

Fig. 4. Automated ultra-thin-layer agarose gel electrophoresis separation of ΦX174 *Hae*III digest restriction fragment mixture. Labeling of the dsDNA fragments was accomplished by TO-PRO-3 intercalation 'on-the-fly' during electrophoresis. Conditions are the same as in Fig. 3, but the separation gel contained 2 n*M* TO-PRO-3. Injection: 5 ng total DNA/lane.

time, 15 min) suggests a mutation in position 1388 on the 21-hydroxylase gene (Table 1, second row). In both PCR reactions, a primer to form a 1263-bp fragment, independent of any mutations, was included as control of the reaction (Table 1, third row). This control fragment appears to be weaker in the sample of Patient-1 compared to the Patient-2 sample, but still recognizable (migration time, 20 min). The non-uniform migration time differences between the individual components of the 100-bp ladder standard are probably due to variations in the complexation with the TO-PRO-3 staining.

4. Conclusion

Ultra-thin-layer agarose gel electrophoresis, a combination of the established methodologies of slab gel electrophoresis and capillary electrophoresis provided advantages such as multilane separation format, rapid analysis time and high separation efficiency. The automated ultra-thin-layer gel electrophoresis–LIF detection system, described in this paper, was used to study known point mutations, in conjunction with the PCR-based ASA method. The resulting PCR products, corresponding to individual mutations, were labeled with the intercalator dye TO-PRO-3 during the separation and detected in real-time by LIF. The limit of detection (LOD) was 0.5 fmol of TO-PRO-3-labeled DNA fragments and the relative standard deviation (R.S.D.) of the lane-to-lane migration time reproducibility was less than 2%, which values are comparable that typical of capillary electrophoresis. Several point mutations were analyzed on the 21-hydroxylase genes of patients with congenital adrenal hyperplasia and the genetic basis for the disease was proven using allele-specific primers and an additional control primer in the PCR. The high throughput, multilane fashion of the system in conjunction with the separation speed, real-time detection and 'on the fly' labeling by a fluorescent intercalator dye provided an easy to use and very sensitive dsDNA analysis method. Multiplexing the allele-specific PCR reactions would further increase the throughput of the system.

Table 1
Primers for CYP-21 mutation analysis by allele-specific amplification

Positions	Product length	CYP-21 mutation	Primers
700–1022	322	1004(T→A)	WT: CCG AAG GTG AGG TAA CAG A Mut: CGA AGG TGA GGT AAC AGT
700–1405	705	1388(T→A)	WT: GCC TCA GCT GCA TCT CCA Mut: GCC TCA GCT GCA TCT CCT
700–1963	1263	Control	Sense: GGA GCA ATA AAG GAG AAA CTG A

Antisense primer for all sense primers: CCT GTC CTT GGG AGA CTA CT.

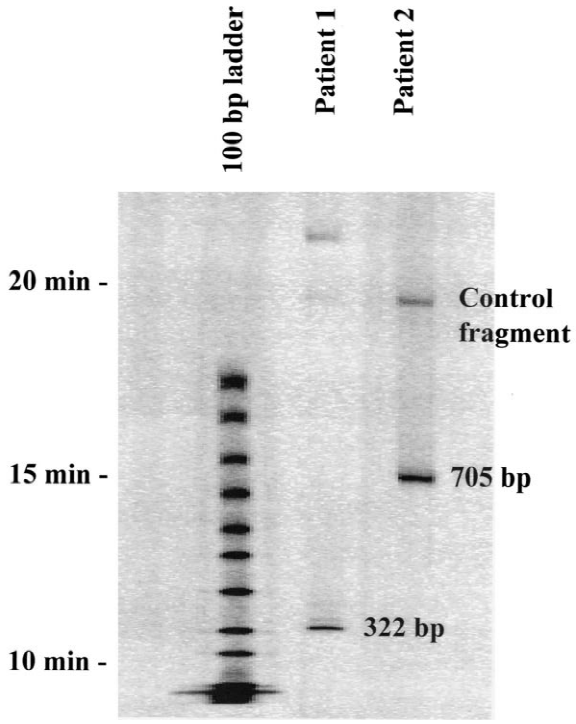

Fig. 5. Screening of mutations on the CYP-21 gene by allele-specific amplification and automated ultra-thin-layer agarose gel electrophoresis. Genomic DNA was prepared from the blood of patients having congenital adrenal hyperplasia. The initial PCR was followed by an allele-specific amplification of DNA fragments, specific for the various disease causing mutations (Table 1). The PCR reaction products were diluted 10-fold and 0.5 μl of the diluted reaction mixture were applied onto the tabs of the membrane loader. For fragment size evaluation a 100-bp ladder was also injected. Conditions were the same as in Fig. 4.

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