Multiplex Capillary Denaturing High-Performance Liquid Chromatography with Laser-Induced Fluorescence Detection

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W. Xiao, D. Stern¹, M. Jain, C.G. Huber², and P.J. Oefner
Stanford University, Palo Alto, CA, ¹Affymetrix, Santa Clara, CA, USA, and ²Leopold-Franzens-University, Innsbruck, Austria

INTRODUCTION

Because of its high sensitivity (>96%) and specificity (>99%) in the detection of mutations in DNA fragments as large as 1000 bp (2,7,9,14, 20,24), denaturing high-performance liquid chromatography (DHPLC) is widely used in the analysis of DNA sequence variation (<http://insertion.stanford.edu/pub.html>). Applications of DHPLC include, among others, the discovery of simple sequence polymorphisms (1,23), the mapping of genes (19,21), the mutational analysis of candidate genes (3,17,24), and the targeted screening for induced mutations (12).

Mutational analysis with partially denaturing HPLC is based on the principle that mismatched base pairing creates double-stranded heteroduplex species with different conformational structure and melting properties from perfectly matched homoduplexes. Under conditions of partial denaturation, this gives rise to different retention of the double-stranded heteroduplexes and homoduplexes using ion-pair reversed-phase HPLC (IP-RP-HPLC), with heteroduplex species typically eluting before the homoduplex species (16). Ideally, as many as four peaks appear in the chromatogram, representing each of the four hybridized species.

ABSTRACT

Denaturing high-performance liquid chromatography (DHPLC) is a sensitive, robust, and operationally inexpensive method for the detection of single-base substitutions and small deletions and insertions. To increase sample throughput, we have developed a multiplexing strategy using fluorophores to distinguish different PCR products. The system combines recent advances in the synthesis of monolithic poly(styrene-divinylbenzene) capillary columns with four-color confocal argon ion laser-induced fluorescence detection. Depending on the change in retention caused by the fluorophores, adjustments in the analysis temperature may be required to ensure the maximum mutation detection sensitivity.

Figure 1. Separation of unlabeled and fluorescent dye-labeled (HEX) 393-bp homoduplex and heteroduplex species. Chromatograms show the analyses of a monomorphic control (a) and four different mismatches (b–e) located at nucleotide positions 73 (TC), 161 (GA), 246 (CT), and 289 (TC), respectively. DHPLC was carried out at 56°C, with a 50 × 4.6 mm i.d. stainless steel column packed with nonporous 2-μm PS/DVB-C18 particles, by increasing the relative concentration of buffer B from 50% to 55% in 0.5 min and from 55% to 64.9% in 5 min. The UV absorbance detection was performed at 254 nm, while the excitation and the emission wavelengths for fluorescence detection were set at 520 and 550 nm, respectively.

As various genome projects that aim at large-scale discovery and the genotyping of single-nucleotide polymorphisms (SNPs) are well underway, there are compelling needs to further develop DHPLC into a high-throughput technique for both the discovery and the genotyping of SNPs (12,21). Recently, it has been demonstrated that using monolithic capillary columns synthesized *in situ* by copolymerization of styrene and divinylbenzene yields high resolving power of ss and dsDNA, which compares well to the resolving power obtained previously with micropellicular poly(styrene-divinylbenzene) (PS/DVB) (18). As a consequence of improved concentration sensitivity offered by the capillary format, it is now possible to combine DHPLC with laser-induced fluorescence detection similar to capillary electrophoresis in DNA se-

quencing (5,10,22). The present study demonstrates the successful, multiplex capillary DHPLC analysis of PCR products labeled with fluorophores of four different colors. It also describes the impact of various fluorophores on retention and melting behavior.

MATERIALS AND METHODS

DNA Sequence and Dye-Labeled Primers

We studied the following two human Y-chromosome sequences containing four and one SNP, respectively. The first one was 393 bp and was amplified with the following forward and reverse primers: 5'-TGGTAACTCTACTTAG TTGCCTTT-3' and 5'-CAGCGAATTA GATTTTCTTGC-3'. The forward pri-

mer was labeled with HEX. The four mismatches were located at nucleotide positions 73 (TC), 161 (GA), 246 (CT), and 289 (TC), respectively, from the 5' end of the product. The second product was 209 bp, and the following forward and reverse primers were used: 5'-AGG CACTGGTCAGAATGAAG-3' and 5'-AATGGAAAATCAGCAGCTCCCC-3'. The single mismatch was located at nucleotide position 168 (AG). The forward primer was labeled with different fluorophores. Oligonucleotides labeled with HEX [emission maximum (12), 555 nm], TET (545 nm), and NED (580 nm), were obtained from Applied Biosystems (Foster City, CA, USA). Unlabeled primers labeled with 6-Fam (520 nm), TAMRA (580 nm), ROX (605 nm), BODIPY FL (515 nm), and BODIPY R6G (550 nm) were obtained from Life Technologies (Rockville, MD, USA).

Figure 2. Impact of different fluorophores on the separation of the heteroduplex and homoduplex components of a 209-bp PCR product with a single mismatch at nucleotide position 168 (AG). DHPLC was performed using a monolithic PS/DVB column, 50×0.2 mm i.d., with a gradient from 47% to 67% of buffer B in 12 min; flow rate, $3.0 \mu\text{L}/\text{min}$. Shown in panel a, fluorescent dye-labeled PCR products were separated at 58°C and detected using the confocal fluorescence capillary array scanner. Excitation was accomplished with an argon ion laser, and emission was recorded at 525, 555, 580, and greater than 595 nm, respectively. Chromatograms obtained for an unlabeled sample at temperatures ranging from 57°C to 62°C , detected by UV absorbance, are displayed in panel b. Because of the increasing hydrophobic nature of the fluorophores studied, increasing concentrations of acetonitrile are required for their elution. Because acetonitrile decreases the melting temperature, peak profiles obtained for some of the more hydrophobic fluorophores resemble those observed for an unlabeled product at higher column temperatures.

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PCR

Each allele was amplified separately in a 50- μ L PCR that contained 1 U AmpliTaq Gold[®] DNA polymerase (Applied Biosystems), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.1 mM each of the four dNTPs, 0.2 μ M each forward/reverse primers, and 50 ng genomic DNA. The thermocycling protocol comprised an initial denaturation at 95°C for 10 min to activate AmpliTaq Gold, 14 cycles of denaturation at 94°C for 20 s, primer annealing at 63°C–56°C with 0.5°C decrements, extension at 72°C for 1 min, followed by 20 cycles at 94°C for 20 s, 56°C for 1 min, 72°C for 1 min, and a final 5-min extension at 72°C. PCR yields were determined semiquantitatively on ethidium bromide-stained agarose gels. For DHPLC analysis, unpurified PCR products of each allele were mixed at an equimolar ratio and

subjected to a 3-min 95°C denaturing step, followed by gradual reannealing from 95°C to 65°C over 30 min.

DHPLC

The capillary HPLC instrument and the monolithic column have been described previously (18). The system consists of a pump (Maxi-Star K-1000; Knauer, Berlin, Germany), a degasser (model DG-1210; Uniflows, Tokyo, Japan), a column oven (Mistral; Varian, Walnut Creek, CA, USA), a four-position microscale injector (model 7410; Rheodyne, Rohnert Park, CA, USA) with a 1- μ L internal sample loop, a laser-induced fluorescence scanner, and a PC-based data system. A T-piece is used to split the primary flow and deliver gradients to the column.

To test the impact of fluorescent dye on the separation of homo- and heteroduplex species, a column packed

with alkylated, nonporous 2- μ m PS/DVB particles (6) was used on a ProStar Helix HPLC system (Varian) equipped with a spectrofluorometer (model FP-920; Jasco, Easton, MD, USA).

The composition of the buffers is (A) 100 mM TEAA, pH 7.0, 0.1 mM Na₄EDTA and (B) 100 mM TEAA, pH 7.0, 0.1 mM Na₄EDTA, 25% acetonitrile. The gradient profiles used for the separations are given in the figure legends.

Laser-Induced Fluorescence Detector

The confocal fluorescence capillary array detector was constructed according to the original scanner by Kheterpa et al. (8), designed for the purpose of capillary array electrophoresis-based DNA sequencing. This scanner collects four-color fluorescence data up to 25 capillaries in parallel. Briefly, capillaries are mounted onto a translation stage driven

by a computer-controlled, microstepping indexer (Zeta6104; Parker Hannifin, Cleveland, OH, USA). An excitation beam (488 nm) from an argon ion laser is focused into the capillary array through a microscope objective. Fluorescence from the capillaries is collected by the same objective and fractioned successively by four longpass dichroic beam splitters (Omega Optical, Brattleboro, VT, USA) with transmission wavelengths (>50% T) of 505, 540, 570, and 595 nm, respectively. Bandpass filters (OMIGA™ 525DF30, 555DF30, and 585DF20) are used on the light reflected by each of the first three beam splitters and a 595-nm longpass filter after the last beam splitter. Filtered light in each fraction is then detected by a photomultiplier tube (model HC120-05; Hamamatsu, Bridgewater, NJ, USA). Signals are lowpass filtered by 4-pole Bessel filters (model 824L8L-4; Frequency Devices, Haverhill, MA, USA)

and digitized by a 16-bit data acquisition board (CIO-DAS1402/16; Computer Boards, Middleboro, MA, USA). Four-color data are filtered using a five-point filter and reduced using color-separation matrices. A computer program synchronizes the movement of the translation stage and data acquisition, allowing each capillary to be scanned twice per second.

RESULTS AND DISCUSSION

Effect of Fluorescent Dye on Heteroduplex Detection

High-resolution liquid chromatography of fluorescent dye-labeled nucleic acids has been discussed in detail by Oefner et al. (15). The sensitivity of fluorescence detection was shown to be 30–100-fold higher than that of UV absorbance detection. The measurement of fluorescence from primer-labeled PCR

products is therefore an attractive alternative for highly sensitive mutation analysis by DHPLC (4). Similar to the sizing of microsatellite markers (11), fluorescent dye-labeling strategies can be used to increase sample throughput by using different fluorophores as sample identifiers. Although multiplexing based on differences in product size has been demonstrated (16), the actual gain in throughput is minimal because of the increased analysis time. Figure 1 shows a comparison of chromatograms obtained for unlabeled and fluorescent dye (HEX)-labeled polymorphic PCR products, detected either by UV absorbance or fluorescence. The chromatographic profiles from fluorescence largely resemble the ones from UV absorbance, which corroborates the feasibility of employing fluorescence-based detection in DHPLC analysis. The slight differences in the peak pattern observed between the chromatograms result mainly

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from the impact of the fluorescent dye on retention that increases because of the hydrophobic nature of the fluorophore. The longer a DNA fragment is retained, the higher the concentration of acetonitrile required for its elution, which in turn causes more extensive denaturation of the product. The changes in the peak pattern are therefore similar to those seen with increasing column temperature (16), which is the paramount factor influencing the detection power of DHPLC. Dyes with higher hydrophobicity bind stronger to the column and are expected to have a larger effect on the chromatographic profiles than more hydrophilic dyes. For the analysis with multiplexed PCR products, attention must therefore be paid to select the group of dyes that share a similar retention behavior to ensure maximum mismatch detection sensitivity.

Laser-Induced Fluorescence Detection with Capillary DHPLC

The peak concentration of analyte in the effluent of conventional 4.6-mm i.d. columns has proven too low to allow the use of a laser-induced fluorescence detector. With the 200- μ m i.d. column, peak concentration is now sufficiently high to allow the detection of fluorescent dye-labeled nucleic acids. The four-color confocal fluorescence capillary array scanner with channels centered at 525, 555, 580, and greater than 595 nm was coupled with capillary HPLC, and different dyes linked to 20-mer oligonucleotides were tested for detection limit and retention time. Using the 488-nm laser excitation, all the fluorescent dyes had comparable emission levels detectable by the scanner (the lowest from ROX is about 50% of the highest from FAM). The lower detection limit was about 0.5 fmol for the dye-labeled 20-mer oligonucleotides. The affinity to column determined by retention time, is, in increasing order, FAM, TET, HEX, TAMRA, NED, BODIPY FL, ROX, and BODIPY R6G. The impact of the different fluorophores on the chromatographic profile of a 209-bp polymorphic PCR product containing a single mismatch (AG) was determined by running all samples under identical gradient and temperature conditions. A comparison of Figure 2, pan-

els a and b, shows that the FAM-labeled PCR product yields a heteroduplex profile virtually identical to that of an unlabeled product. All other fluorophores led to noticeable increases in retention. Chromatographic profiles obtained for TET- and HEX-labeled products were quite similar to that of FAM, but the peak patterns obtained with NED and TAMRA more closely resembled that of an unlabeled product analyzed at 59°C. The products labeled with ROX and the BODIPY dyes, which were retained the strongest, resembled an unlabeled product analyzed at 60°C.

Figure 3 shows the multiplex detec-

tion of PCR products labeled with four different fluorescent dyes (FAM, HEX, NED, and ROX, respectively) that have been used widely in four-color DNA sequencing. The four products were generated separately and mixed together before injection, separated by the capillary column, and detected by the four-color confocal fluorescence capillary array scanner. Species tagged with different dyes are identified by the characteristic emission spectrum of each attached fluorophore measured in the four channels of the detector, as shown in Figure 3, panels a–c. The monomorphic PCR products, yielding single peaks

Figure 3. Capillary DHPLC and laser-induced fluorescence detection of a single-base mismatch in a 209-bp product tagged with FAM (peak 1), HEX (peak 2), NED (peak 3), and ROX (peak 4). Color-separated traces are shown in panel a for the A allele, in panel b for the G allele, and in panel c for the heterozygote. Column, monolithic PS/DVB, 50 $\times$ 0.2 mm i.d.; linear gradient, 50%–70% B in 10 min; flow rate, 3.0 μ L/min; temperature, 58°C.

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only (Figure 3, panels a and b), are clearly distinguished from the polymorphic fragments (Figure 3, panel c) by their distinct multiple peaks. However, variations in peak profile are present among the four polymorphic products because they differ in retention behavior as a function of the fluorophore attached. The differences among FAM, HEX, and NED are small enough not to significantly affect the chromatographic profile (i.e., the heterozygote shows four peaks in the chromatogram). In contrast, because of its more hydrophobic nature, the rhodamine dye ROX elutes at a higher acetonitrile concentration than the other three dye-labeled fragments; as a consequence, denaturation sets in at a temperature 2°C lower. This may affect the detection sensitivity of multiplex DHPLC experiments for certain mutants for which peaks from heterozygotes only show up in a narrow temperature range (24). One possible solution is to include in the mixture a PCR product with a higher melting temperature, as determined by the melting algorithm described previously (7). Alternatively, changes in the filter combinations used may allow the use of TET as a fourth fluorophore, which has similar retention characteristics to FAM, HEX, and NED and whose emission maximum is at 545 nm.

In conclusion, fluorescence detection provides a highly specific and sensitive method for polymorphism detection with DHPLC. It has been demonstrated that multiplexed SNP analysis can be achieved with four-color measurements on samples labeled with different dye molecules. Because the same detector can scan up to 25 capillaries, we are in the process of developing a capillary array HPLC system to mimic existing capillary array electrophoresis systems for DNA sequencing (5) and sizing of microsatellites (11), which will result in an approximately 100-fold increase in throughput over today's commercially available DHPLC systems.

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Address correspondence to:

Dr. Peter J. Oefner
Stanford Genome Technology Center
855 California Avenue
Palo Alto, CA 94304, USA
e-mail: oefner@genome.stanford.edu