Denaturing high performance liquid chromatography in the molecular diagnosis of genetic disorders

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With the availability of a draft human genome sequence and the increasing number of monogenic disorders and polymorphisms identified that confer susceptibility to common multifactorial disorders, there arises a need for high throughput methods for mutation/polymorphism detection. Among the methods available for detecting DNA sequence variation, Denaturing High Performance Liquid Chromatography (DHPLC) has emerged as the most sensitive method that provides a high degree of automation and throughput. DHPLC detects successfully single-nucleotide substitutions, small deletions and insertions by on-line UV or fluorescence monitoring within 2–5 min in unpurified amplicons as large as 1.5 kb. Apart from mutational analysis, DHPLC can be applied to genotyping, LOH determination and gene expression analysis. The low operational cost and broad applicability in pre- and postnatal diagnosis of genetic disorders make DHPLC a worthwhile investment, despite the high initial procurement cost.

THE exponential increase in the discovery of genes over the past few years has transformed the DNA diagnosis of genetic disorders from a minor research-based activity to a major professional operation. For any genetic disease, once the defective gene is identified, knowledge of the pathogenic mutations is indispensable to offer DNA diagnosis. DNA diagnosis is offered at pre- and postnatal levels either by direct or indirect approaches. Direct mutational analysis and linkage studies with highly polymorphic intragenic markers are carried out depending on the feasibility of their detection. This is not as easy as it may sound, because many genes, including the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene, the breast cancer genes (*BRCA1* and *BRCA2*), and the retinoblastoma gene (*RBI*), lack mutational hot spots necessitating an exhaustive analysis of coding and flanking intronic and regulatory sequences.

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Apart from the genes that cause monogenic disorders, the discovery of functional sequence variants that confer genetic susceptibility to common multifactorial disorders, such as cardiovascular disease, psychiatric disorders, autoimmune disorders and cancer, also creates demand for high throughput testing for clinically relevant polymorphisms. Single Nucleotide Polymorphisms (SNPs) are the most abundant type of DNA sequence variations in the human genome[1], though only miniscule fraction causes significant changes in amino acid sequence. One area where SNPs may have an immediate impact on patient care, is the individual response to drug therapy, commonly referred to as pharmacogenetics. A genetic variability in *N*-acetyl transferase (NAT-2), for instance, is associated with a high incidence of peripheral neuropathy when taking isoniazid, an antituberculosis drug[2]. A variant in the core promoter of the *ALOX5* gene, on the other hand, is responsible for the failure of some asthma patients to respond to treatment with ALOX5-pathway modifiers[3].

The ultimate method for detection and definition of mutations is direct sequencing. But it comes at significant cost and labour, and the vast majority of sequencing reactions will only exclude the presence of a mutation. This has led to the development of physical, chemical and biological mutation screening methods[4], that exclude the presence of mutations at a fraction of the cost of sequencing, but provide little, if any, information on the location and nature of sequence variation in mutated gene fragments. Most methods do well with regard to specificity, but fail miserably when it comes to sensitivity. They include such popular methods as single-strand conformation analysis, gel electrophoresis-based heteroduplex analysis, and denaturing gradient gel electrophoresis[5]. Other methods, including the Protein Truncation Test detect only certain classes of sequence variants such as protein-truncating mutations. Hence, an ideal method for mutation screening should match direct sequencing in sensitivity, lack any bias for certain mutations, and be highly automated and offer high sample throughput. Denaturing HPLC, which was developed in 1995, meets all the aforementioned criteria[6–8].

Principle of DHPLC

Denaturing HPLC detects mutations on the basis of mismatches between amplified chromosomal fragments that result in the formation of heteroduplices[8]. The heteroduplices, which are thermally less stable than their corresponding homoduplices, are resolved by means of ion-pair reversed-phase liquid chromatography at elevated column temperatures typically in the range of 50–70°C depending on the GC-content of the sequences. Key to sustained performance is a physically and chemically inert stationary phase consisting of alkylated nonporous poly (styrene-divinylbenzene) particles of 2–3 microns in diameter[9], commercially available under the brand name DNA Sep™ from Transgenomic Inc., San Jose, CA, USA. The alkylated stationary phase is electrically neutral and hydrophobic. DNA, with its phosphate group, is negatively charged and therefore cannot adsorb to the column's matrix by itself. A binding molecule, also known as ion-pairing reagent, is needed to help the adsorption of DNA to the stationary phase. Triethlyammonium acetate (TEAA) is used for this binding process. The positively charged ammonium ions of the TEAA molecules interact with the negatively charged phosphate ions of DNA molecules, while the alkyl chains of the TEAA molecule interact with the hydrophobic surface of the stationary phase. The separation of DNA molecules is achieved mainly by the electrostatic interactions between the positive surface potential generated by triethylammonium ions and the negative surface potential generated by the dissociated phosphodiester groups of DNA. Therefore, double-stranded DNA (dsDNA) molecules are retained according to their chain length (Figure 1). Additional solvophobic interactions are minimal in the case of dsDNA molecules because of their highly hydrophilic outer surface. The absorbed DNA molecules are eluted by an increase in the concentration of organic solvent, acetonitrile, in the mobile phase, which results in desorption of the amphiphilic ions and DNA molecules. The eluting DNA fragments are typically detected by UV absorbance at 254 nm, thereby eliminating the tedious process of staining slab gels.

The retention behaviour of dsDNA changes with the column temperature. At temperatures < 50°C, the elution order is strictly related to the length of the dsDNA molecule[10]. At temperatures > 50°C, however, the dsDNA molecules begin to denature, i.e. to disintegrate into their complementary single-strand components. The process is facilitated by the presence of acetonitrile in the mobile phase, which may be the cause of variability in elution profiles among columns, as differences in surface area require different concentrations of acetonitrile to elute the hetero- and homoduplices. Fortunately, the effect of acetonitrile on denaturation can be counteracted by varying column temperature[11] allowing to harmonize elution profiles from column to column with the help of mutation standards that yield well-defined chromatograms at specific temperatures. Such mutation standards are now commercially available from Transgenomic Inc.

The presence of base mismatches causes heteroduplices to denature more extensively than their corresponding perfectly matched homoduplices. As a consequence, they are retained less and elute in front of the homoduplices as one or more additional peaks depending on the nature and number of mismatches. Prerequisite for successful mutation detection is the formation of heteroduplices prior to DHPLC analysis. For that purpose, wild type and mutant chromosomal fragments are typically mixed in a ratio of 1 : 1. Fragments are then denatured at 95°C for 3 min, before they are allowed to renature slowly over 30 min by decreasing the temperature to 65°C. During renaturation, not only are original homoduplices formed, but also heteroduplices between the sense and antisense strands of either homoduplex. The thermal instability of the heteroduplices and, to a lesser extent of the homoduplices, depends on the influence of the nearest sequence[12] and hydrogen bonding between non-Watson–Crick base oppositions such as GT and GA[13]. Hence, depending on the nature of the mismatch and the sequence of the entire fragments, it may be feasible to separate not only the individual heteroduplices, but also homoduplices (Figure 2). Moreover, different mismatches will result, though not always, in different elution pro-

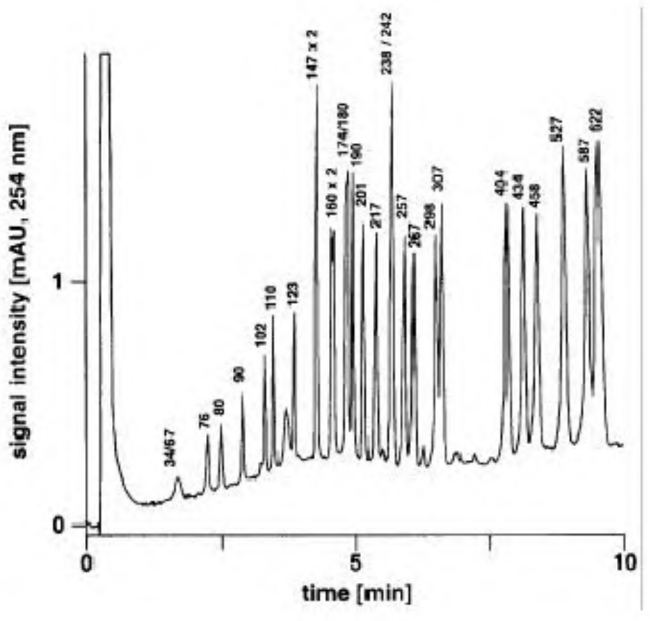

Figure 1. High-resolution separation of double-stranded DNA fragments obtained from *Hae*III and *Msp*I digests of pUC18 and pBR322 respectively, using conventional 4.6 mm i.d. column packed with poly-(styrene-divinylbenzene) microparticles. Conditions in the column: 2 µm PS/DVB-C18 particles (DNA Sep™, Transgenomic Inc.), 50 × 4.6 mm i.d.; mobile phase: (A) 100 mM TEAA, 0.1 mM Na_4EDTA, pH 7.0, (B) 100 mM TEAA, 0.1 mM Na_4EDTA, pH 7.0, 25% acetonitrile; linear gradient: 38–56% B in 4 min, 56–67% B in 6 min; flow-rate: 0.90 ml/min; column temperature: 50°C; injection volume: 8 µl.

files (Figure 3). In practice, any change in elution profile, including such subtle changes as the appearance of a fronting or trailing shoulder, is diagnostic and warrants subsequencing to establish the location and nature of the variant base(s).

Sensitivity and specificity of DHPLC in detecting mutations

Temperature is the most important parameter that affects the sensitivity of DHPLC in detecting mutations. Originally, the optimum temperature for the analysis of a particular DNA sequence was determined empirically by injecting repeatedly a test sample at gradually increasing column temperatures until the duplex product peak begins to shift signficantly (~ 1 min) towards shorter retention times. At that point, the presence of mismatch(es) will be usually detected by the appearance of one or two additional peaks eluting immediately before the homoduplex signals. However, this empirical approach of determining the appropriate temperature of analysis, harbours the risk that mismatches in low-melting AT-rich domains may go undetected due to complete denaturation. Further, it impedes automation and sample throughput. For these reasons, an algorithm was developed that calculates for every site in a known sequence the probability that 50% of the fragments are closed[14]; it is freely available at the website http://insertion.stanford.edu/melt.html.

The first gene subjected to DHPLC analysis was the calcium channel gene *CACNL1A4* (ref. 15). To date, more than hundred genes have been analysed and the excellent sensitivity and specificity of DHPLC in detecting small mutations are documented in the literature (http://insertion.stanford.edu/pub.html). In a blind analysis of detecting mutations in the exon H of the Factor IX blood coagulation gene (*F9*) and exon 16 of the neurofibromatosis type 1 gene (*NF1*), O'Donovan *et al.*[16] reported a sensitivity and specificity of 100% respectively. Previous analysis of exon H of *F9* gene by Single Strand Conformation Polymorphism (SSCP) analysis had detected only 50% of the mutations[17]. Direct sequence analysis of 626 *BRCA1* fragments previously subjected to DHPLC analysis confirmed the 100% sensitivity and specificity of DHPLC in detecting the mutations[18]. Wagner *et al.*[19] tested 180 different mutations in *BRCA1* and *BRCA2* genes by DHPLC and found distinct elution profiles in 179 of the 180 mutations. The concomitant blind analysis of *BRCA1* gene in 41 index cases showed that four putatively disease-causing mutations were identified by DHPLC, while only three of those four sites were detected by Denaturing Gradient Gel Electrophoresis (DGGE)[19]. Jones *et al.*[14], while scanning CFTR, *TSC1* and *TSC2* (Tuberous sclerosis) genes, found that DHPLC

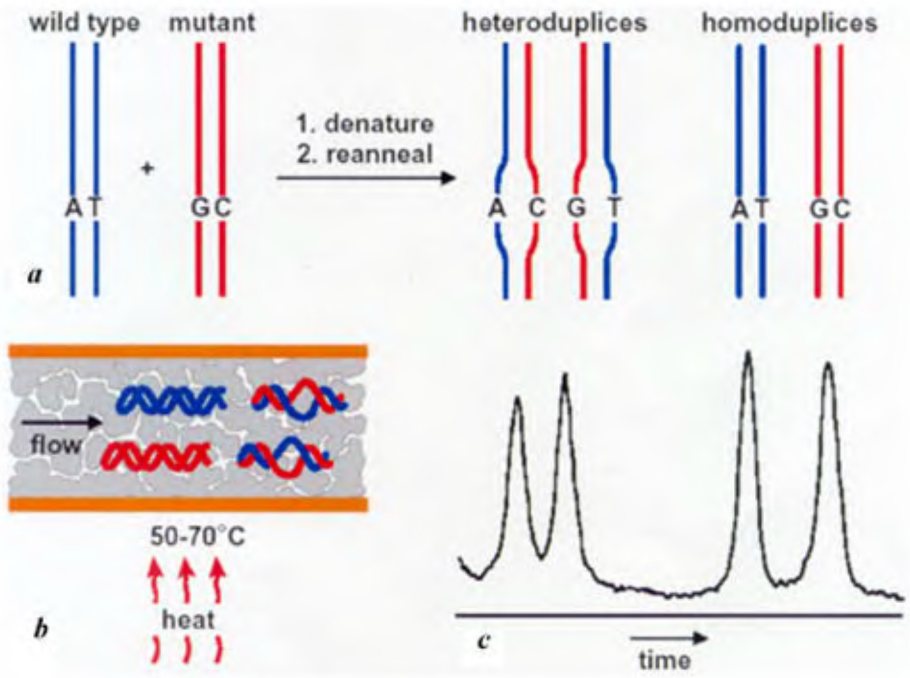

Figure 2. Principle of mutation detection by DHPLC. ***a***, DHPLC compares typically two chromosomes as a mixture of PCR products denatured at 95°C for 3 min, and re-annealed over 30 min by gradual cooling from 95 to 65°C prior to analysis. In the presence of mismatch, not only are the original homoduplices formed, but also the sense and antisense strands of either homoduplex form heteroduplices; ***b***, Heteroduplices denature more extensively at the analysis temperature (ranges from 50 to 70°C) and are eluted earlier than the homoduplices in the DNA Sep column; ***c***, Corresponding chromatographic pattern shows four different peaks belonging to four different species of DNA.

detected 96% of all the mutations in PCR products varying in size from 173 to 630 bp, in comparison to SSCP that detected only 85% of the mutations. In another study on *BRCA1*, the sensitivity and specificity of DHPLC and SSCP were reported to be 100 and 94% respectively[20].

The sensitivity of SSCP drops sharply for fragments larger than 300 base pairs[21] or in the case of mutations located within hairpin-like structures[22]. In contrast, the sensitivity of DHPLC does not drop over a size of 150–700 bp[16,19]. No sequence motif has been identified to date that impedes the detection of mutations by DHPLC. This is also true for GC-rich sequences. Escary *et al.*[23] succeeded in detecting 97% of all mutations in the *Notch 3* gene despite its high GC content of 66%, after establishing theoretical optical parameters. In addition, DHPLC allowed identification of two novel pathogenic NOTCH3 mutations and a novel polymorphism previously missed by SSCP and gel-based heteroduplex analysis. DHPLC is also less affected than DGGE by the presence of different melting domains in a fragment. DHPLC analysis of exon 8 of the *HPRT* gene detected 20/20 mutations irrespective of their location in either of the two domains with melting temperatures of 65 and 69°C respectively. In contrast, DGGE failed to detect mutations in the higher melting domain[24].

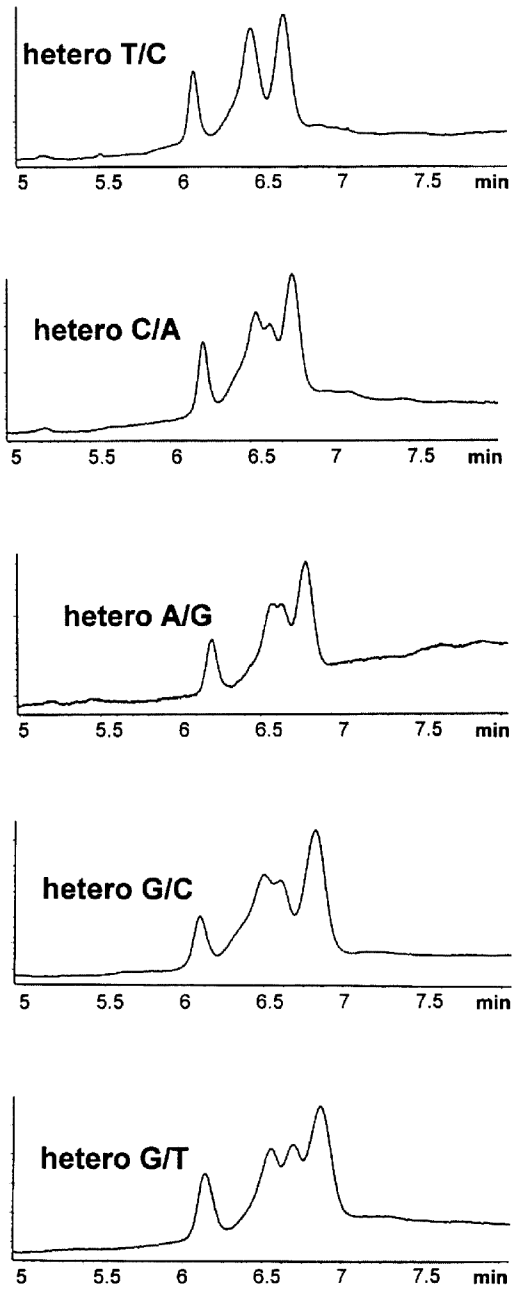

Figure 3. Separation of five different mismatches contained in homologous 1000-base pair amplicons. Column: 50 × 4.6 mm i.d. (DNA Sep™, Transgenomic Inc.,); eluant A: 0.1 M TEAA, pH 7.0, 0.1 mM Na_4EDTA; eluant B: 0.1 M TEAA, pH 7.0, 25% acetonitrile; gradient: 40–62% B in 3 min, 62–72% B in 5 min; flow-rate; 1.0 ml/min; column temperature: 57°C; detection: UV 254 nm.

We recently analysed the entire coding, flanking, intronic and promoter regions of *RB1* gene in 19 patients with hereditary retinoblastoma with DHPLC followed by DNA sequencing. We were able to identify mutations in nine out of these 19 patients[25]. In order to evaluate the sensitivity of DHPLC in the detection of *RB1* gene mutations, all the products that were detected as homozygous by DHPLC were re-analysed by dye terminator sequencing. Sequencing identified no additional sequence changes.

Direct sequencing is considered to be the golden standard in mutational analysis. However, direct sequencing fails to detect mutant alleles present at low frequency, such as in somatic mosaicism[26]. DHPLC, in contrast, detects mutant alleles reliably at frequencies as low as 10% (ref. 27). Hence, reproducible heteroduplex profiles should not be classified prematurely as false positives, if direct sequencing of the PCR products fails to identifty a mismatch. The amplified fragments should be rather cloned, followed by sequencing of a sufficient number of clones.

In the case of screening for homozygous mutations, the test samples should be mixed with a known homozygous sample, in order to generate homo- and heteroduplices. Recently, Lin *et al.*[28] applied DHPLC to the detection of mutations in the *GJB2* gene in both homo- and heterozygous carriers. DHPLC identified all 38 sequence variations, which had been initially detected by direct sequencing, thus confirming the 100% sensitivity and specificity of DHPLC in detecting connexin 26 mutations that are responsible for a majority of congenital, hereditary hearing impairment.

Completely denaturing HPLC

Mutational analysis in shorter DNA fragments of size 50–100 bp is performed under completely denaturing conditions by DHPLC. The high resolving power of the separation system makes it possible to discriminate two single-stranded nucleic acids of identical size with a dif-

ference in base composition as small as a single base out of one hundred bases[20]. Under completely denaturing conditions, the alleles of a given polymorphic locus can be resolved without the addition of a reference chromosome, as retention times are allele-specific. Completely denaturing HPLC has been also been applied to the analysis of mini-sequencing or primer extension reactions[30]. Briefly, primer extension products are prepared by PCR amplification of the DNA region containing the SNP of interest. Subsequently, an oligonucleotide primer is annealed immediately upstream or downstream from the polymorphism. In the presence of the appropriate dNTPs and ddNTPs, the primer is extended by one or more bases depending upon the sequence at the polymorphic site. The alleles are then distinguished by completely denaturing HPLC on the basis of either size of the extended product or differences in retention depending on the ddNTP incorporated. The DHPLC-based analysis of primer extension reactions is a cost-effective alternative to mass spectrometry-based analysis[31], if the number of samples is only in the hundreds.

Multiplex DHPLC analysis

In contrast to capillary electrophoresis, conventional HPLC has not lent itself readily to parallel analysis using an array of columns. But with the recent introduction of monolithic poly(styrene-divinylbenzene) capillary columns, it is now possible to analyse multiple samples in parallel[11]. The throughput increases with the number of columns used in the systems, and a further enhancement in the number of samples analysed simultaneously is achieved by the combination of this technology with fluorescent colour multiplexing[32]. Different amplicons are labelled with different fluorophores during PCR using fluorescent dye-labelled primers. The PCR reactions are pooled together, after having been denatured and re-annealed separately, and analysed simultaneously in one chromatographic column. Elution of the differentially-labelled fragments is monitored at emission wave lengths characteristic to the fluorophores employed. Since the fluorescent dyes affect retention of the labelled nucleic acids to various degrees, the same polymorphic amplicon labelled with different fluorophores will yield different heteroduplex profiles. The capillary array HPLC has been successfully applied to detect the mismatches under both partially and completely denaturing conditions[11,33].

Other applications of DHPLC

In addition to mutation detection, applications of DHPLC include genotyping of Short Tandem Repeats (STRs), determination of Loss of Heterozygosity (LOH) in tumour samples, and measurement of changes in gene expression by quantitative RT–PCR.

Genotyping of STRs

Genotyping of STRs is based on amplification of the fragments of interest, generating products of different sizes corresponding to the different alleles. When the products are analysed by DHPLC, individual dsDNA fragments elute off the column according to their size and discrimination of 2-bp differences can be accomplished up to 300 bp[34,35].

Determination of LOH

The presence or absence of PCR products that can differentiate between the two alleles of the gene is the basis for LOH analysis by DHPLC. This is done by comparing the elution profiles of amplified DNA from both normal and tumour tissue. The allele ratio (AR), calculated by using areas under the peaks for each allele, for the constitutive heterozygous condition is compared with the AR of the tumour. If the tumour AR is significantly larger than the mean AR of the heterozygous condition, it is interpreted as the LOH of the tested gene in the tumour. Using this approach, Klyemenova and Walker[36] and Gross *et al.*[37] conducted LOH analysis for loss of the normal *Tsc-2* allele in renal cell carcinoma (RCC) arising in Eker rats and loss of *p53* gene in ovarian tumours respectively.

Quantitative analysis of gene expression

An important application of DHPLC has been the quantitative analysis of competitive reverse transcriptase polymerase chain reactions (RT–PCR) for the quantification of gene expression in micro-dissected tissue samples[38]. Previous attempts to determine quantitatively the amount of native and competitive transcripts by slab gel electrophoresis had failed to identify the heteroduplices formed between the mixed strands of native and competitor amplicons, and resulted in inaccurate gene quantification. However, the heteroduplex molecules are readily separated by DHPLC. This obviated the need for titration of known RNA inputs. It was also demonstrated, using known inputs of native and competitive RNA, that differences in the secondary structure between the native and competitor gave rise to reproducible differences in the reverse transcription efficiency, while they were not found to affect PCR efficiency. The accuracy of competitive RT–PCR in combination with DHPLC makes it particularly attractive for measuring gene expression in low abundance samples such as microdissected nephron segments. In all other instances, the 5′ nuclease assay, commonly referred to as TaqMan™ or real-time PCR, has become the method of choice for the quantitation of gene copies[39].

Conclusions

Apart from its high sensitivity in detecting gene mutations in DNA fragments up to 700 bp in length, DHPLC has a number of marked advantages over other screening methods. DHPLC is automated and has obviated the need for preparing and loading electrophoresis gels. The analysis is fast, enabling the analysis of more than 200 samples per day on a single instrument. Operational cost is about ten times lower than that of bidirectional sequencing[18,40], as columns can be used routinely for 5000 analyses without significant changes in separation efficiency. Although the initial capital investment in procuring the DHPLC machinery is significant, its tested superiority over other screening methods justifies and calls for its use in pre- and postnatal diagnosis of genetic diseases.

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