With an increasing number of genome sequences completed, denaturing high-performance liquid chromatography (DHPLC) has increasingly established itself as one of the most powerful tools for DNA variation screening and allele discrimination. Aside from its obvious application in identifying mutations in various diseases, it has provided unprecedented insight into human evolution based on the screening of Y chromosome and mitochondrial DNA sequences. It has been applied successfully to the mapping and cloning of genes in yeast, Arabidopsis thaliana, fruit fly, and mouse, and has been implemented for the quantitative measurement of gene expression and the analysis of single nucleotide extension products.

The detection of mutations in DNA fragments by DHPLC is carried out in one of two different modes, depending on the length of the fragment.\(^1\) Partially denaturing HPLC compares two or more chromosomes as a mixture of denatured and reannealed polymerase chain reaction (PCR) amplicons. Upon reannealing, not only the original homoduplexes are formed again but, simultaneously, the sense and antisense strands of either homoduplex form heteroduplexes in the presence of mismatches that are thermally less stable (Figure 1a, left). The more extensive but still partial denaturation of the heteroduplexes at elevated temperatures, typically in the range of 50–70 °C depending on the GC content of the DNA fragment under investigation, results in their reduced retention on the chromatographic separation matrix. As a consequence, one or more additional peaks appear in the chromatogram, with different mutations yielding distinctively different peak profiles.

Temperature is the most important experimental parameter in determining sensitivity, and its optimum can be predicted by computation. Single-nucleotide substitutions, deletions, and insertions have been detected successfully within 2–3 min in unpurified amplicons approx. 100–1500 bp in length, with sensitivity and specificity of DHPLC consistently approaching 100%.

The second mode of DHPLC is...
These columns have proven different amplicons are labeled with various fluorescence dyes during PCR using dye-labeled primers. The samples are then pooled and analyzed simultaneously in one chromatographic column, and are monitored separately by observing their characteristic emission wavelengths. Figure 1a (right) shows the multiplex detection of four 209-bp PCR products labeled with four different fluorescent dyes. The four products were generated separately and mixed together before injection, separated under partially denaturing conditions in the capillary column, and detected by a fluorescence scanner. The characteristic peak patterns allow the designation of the samples as heterozygotes.

The use of optical detectors limits the fragmentation; the sequence can be reconstructed from the mass spectrum of the generated information content. The higher sample throughput and improved cost effectiveness, the serial analysis does not easily allow the necessary higher sample throughput and improved cost effectiveness, and the use of optical detectors limits the generated information content. The solution to these demands comes with the introduction of monolithic columns in the capillary format with 200 µm i.d. These columns have proven to be at least as efficient and durable as conventional packed columns, and their low flow rate and small sample consumption on the order of a few hundred nanoliters have paved the way for exciting new developments.

The greater concentration sensitivity of the capillary format makes it possible to combine DHPLC with laser-induced fluorescence detection for the analysis of shorter fragments typically 50–100 bp in size that differ in a single or multiple bases (Figure 1b, left). The high resolving power of the chromatographic separation system makes it possible to discriminate two single-stranded nucleic acids of identical size with a difference in base composition as small as a single base out of 100 bases. In this technique, the alleles of a given polymorphic locus can be resolved without the addition of a reference chromosome. The only exception to this rule has been C to G transversions.

Today, DHPLC is predominantly performed on commercially available chromatographic columns in the conventional 4.6-mm-i.d. format packed with nonporous alkylated poly(styrene-divinylbenzene) beads. While this technology offers high sensitivity and productivity, the serial nature of the chromatographic analysis does not easily allow the necessary higher sample throughput and improved cost effectiveness. The solution to these demands comes with the introduction of monolithic columns in the capillary format with 200 µm i.d. These columns have proven to be at least as efficient and durable as conventional packed columns, and their low flow rate and small sample consumption on the order of a few hundred nanoliters have paved the way for exciting new developments.

The greater concentration sensitivity of the capillary format makes it possible to combine DHPLC with laser-induced fluorescence detection for the analysis of single-nucleotide polymorphisms (SNPs) in analogy to capillary electrophoresis in DNA sequencing. In this technique, higher throughput is enabled by color multiplexing. Different amplicons are labeled with various fluorescence dyes during PCR using dye-labeled primers. The samples are then pooled and analyzed simultaneously in one chromatographic column, and are monitored separately by observing their characteristic emission wavelengths. Figure 1a (right) shows the multiplex detection of four 209-bp PCR products labeled with four different fluorescent dyes. The four products were generated separately and mixed together before injection, separated under partially denaturing conditions in the capillary column, and detected by a fluorescence scanner. The characteristic peak patterns allow the designation of the samples as heterozygotes.

High-throughput mutation screening and gene mapping is made possible by bundling monolithic capillary columns into arrays similar to those already used in capillary electrophoresis (Figure 1a, center). Multiple samples can be analyzed simultaneously in different columns at the same or at different temperatures using only one pump, injection, and scanning detection device. The throughput increases with the number of columns used in the system, and a further enhancement in the number of samples analyzed simultaneously is achieved by the combination of this technology with fluorescent color multiplexing. Both multiplexing techniques can be applied to partially and completely denaturing HPLC. The ultimate goal of this development is to analyze a full PCR plate in one single chromatographic run, transforming DHPLC into a true genome scale technology.

Another development enabled by the introduction of monolithic capillary columns is the hyphenation of the chromatographic separation with mass spectrometric detection, which can dramatically increase the information gained from a single analysis. DHPLC in capillary columns is highly suited for the direct coupling to electrospray ionization-mass spectrometry (ESI-MS) due to the volatile nature of the mobile phase components, low flow rate, and on-line removal of cations from nucleic acid samples. The hyphenated techniques allow the analysis of mixtures of nucleic acids found in real matrices such as PCR reactions in a time frame of a few minutes. Very similar DNA fragments that...
cannot be completely resolved by the chromatographic process and elute as one single chromatographic peak can be deconvoluted and their components identified using ESI-MS due to their separate characteristic mass signals. Exact mass measurements permit the positive confirmation of the identity of the resolved components and the detection of deletions and insertions in DNA fragments up to 500 bp, as well as the genotyping of single-nucleotide substitutions in fragments up to 100 bp. Even a C to G transversion that cannot be resolved using completely denaturing HPLC can be detected easily in fragments shorter than 120 bp if DHPLC is combined with mass spectrometry, since the transversion is associated with a shift in mass of the DNA single strands of 40 mass units. This difference in mass can easily be detected taking advantage of the high mass accuracy of modern mass spectrometers.

Finally, the implementation of tandem mass spectrometric techniques will allow the determination of location and nature of base changes as well as the detection of multiple markers in PCR fragments with a size of up to 100 bp (Figure 1b, center and right). In this technique, fragmentation of the DNA is induced in the mass spectrometer, the generated fragments are detected, and the sequence can be reconstructed from the found fragments. Since DHPLC, under completely denaturing conditions, resolves the chromosomal fragments in analogy to cloning, it becomes possible to confirm experimentally the phase of the different SNP alleles on a chromosome. This significantly increases the information content of SNPs and has significantly enhanced our understanding of human evolutionary history.

In conclusion, the introduction of monolithic columns in the capillary format has opened up numerous possibilities to enhance DHPLC, enabling the achievement of the necessary increased sample throughput by multiplexing techniques and providing dramatically enhanced information content by the introduction of mass spectrometric detection techniques.

References