DNA Diagnostics by Capillary Electrophoresis

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1. Introduction

Early molecular methods in clinical genetic diagnostics were frequently regarded as unambiguous and definitive. The power of modern molecular biology was seen as sufficient for providing, in theory at least, a successful genetic analysis. This could even lead to a therapy based on a single gene−single disease concept. However, it has become increasingly clear that genetic approaches alone are insufficient for the satisfactory description and diagnostics of complex phenotypes. Monogenic diseases such as Duchenne muscular dystrophy, hemophilia, or sickle cell anemia represent less than 2% of the total human disease burden. The rest are polygenic diseases and must be viewed in the larger context of a cell or an organism. Even though it is clear that detection of individual molecular genetic markers cannot completely replace a complex diagnostic approach, the importance and necessity for reliable high-throughput analytical technologies and sensitive tools for molecular diagnostics is unquestionable.[1]

Every living organism accumulates changes to its DNA material during its life span. These changes occur in a number of ways: (i) copying errors during replication; (ii) changes caused by the environment (radiation, exogenous chemicals, toxins, hormones, or even diet); (iii) spontaneous DNA damage (depurination, depyrimidination, and deamination), resulting in loss of a nucleotide base or a change in the base-pairing properties of a base. When speaking about DNA mutations or polymorphisms, we mean only the stable changes in the nucleotide sequence of the genome resulting from damage or alterations to the DNA material that have not been corrected. While polymorphism represents changes in the DNA sequence that are present in at least 1% of the population and are not considered harmful, mutations are less common (less than 1% of population) and frequently result in disease or an increased risk for developing a disease. Alternative forms of genes at a particular locus are called alleles. Identical or different alleles at a particular locus in an individual are referred to as homozygous or heterozygous, respectively.[2]

Sequence variations at the DNA level can be characterized as (i) substitutions of a single nucleotide, also called point mutations, (ii) deletions of a single or multiple nucleotides, and (iii) insertions of single or multiple nucleotides, which can also have the character of duplications. Even a single nucleotide deletion or insertion can result in translation of a defective protein or no protein at all. This is due to the fact that the amino acid sequence of a protein is determined by the sequence of three-nucleotide codons. Thus, most insertions and deletions induce a reading frame shift of codon sequence. Sequence variations in noncoding regions do not affect a protein sequence directly but can result in proteins being made at the wrong time, in the wrong cell type, or in altered quantities.

One particular type of polymorphism is called tandem repeat polymorphism, frequently analyzed for medical and forensic purposes as a "barcode" for an individual's DNA identification. In practice, two kinds of these highly poly-

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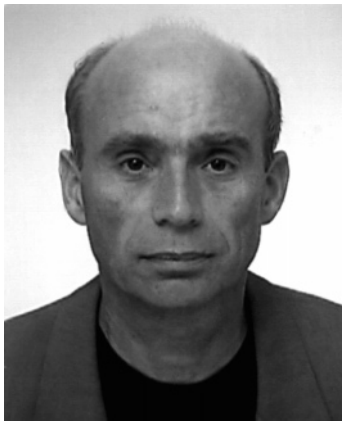


Karel Klepárník received his M.S. degree in Chemical Engineering from the Technical University, Brno, in 1974 and his Ph.D. degree in Analytical Chemistry from the Institute of Analytical Chemistry of the Czechoslovak Academy of Sciences, Brno, in 1987. The subject of his thesis was a theoretical study on the transport phenomena in field-flow fractionation. He has been involved in capillary electrophoresis development since 1990, when he joined Dr. Boček's group. He had active collaboration with Prof. Karger's lab at The Barnett Institute, Northeastern University, Boston, MA, where he worked on high-throughput DNA sequencing in 1995 and DNA differential display in 1997–98. His research interests involve theoretical and experimental aspects of the miniaturization of separation methods and applications of mass spectrometry, laser-induced fluorescence, microfluidic separation devices, and nanotechnology in bioanalytical instrumentation.

Petr Boček was born on December 25, 1941, in Brno, Czechoslovakia. He graduated in Chemistry in 1964 at Masaryk University, Brno, Czechoslovakia, and received his Dr. rer. nat. (RNDr.) in Analytical Chemistry at the same university in 1967. In 1966 he began his work in gas chromatography at the Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, in Brno, and in 1969 he obtained his Ph.D. degree in this field. He then spent 1 year at the University of Eindhoven, The Netherlands, working on column technology for GC. In 1972 he became engaged in analytical isotachophoresis, and after a decade of research, he received his D.Sc. degree in Analytical Chemistry in 1983. He is currently head of a group working on all aspects of analytical electrophoresis at the Institute of Analytical Chemistry in Brno, and for two 4-year terms (from 1993 to 2001) he was head of this institute. He spent two long-term scientific stays at NIH, Bethesda, MD, working in the field of separation of DNA fragments by capillary electrophoresis in 1991 and 1993. In 1996 he was appointed Professor of Analytical Chemistry at the University of Pardubice, Czech Republic. He is the author or coauthor of more than 250 scientific papers and two books titled *Analytical Capillary Isotachophoresis* and *Capillary Zone Electrophoresis*. He is Senior Deputy Editor of the international journal *Electrophoresis* and member of the Editorial Boards of several other scientific journals. For more than 30 years he was a prominent speaker at many national and international scientific meetings, and his presentations always provided new insights.

morphic and repetitive regions are analyzed: minisatellites (regions with a variable number of tandem repeats (VNTR), which consist of units that range from 8 to 50 base pairs in length) and microsatellites (short tandem repeats (STR) with 2−7 base pairs units).

In recent years, increasing interest has been paid to analysis of polymorphism at a single-nucleotide level. This is called single-nucleotide polymorphisms (SNP). SNP analysis is gaining in importance for diagnostic and therapeutic purposes as well as determining the genetic predisposition for certain diseases. A SNP can result in either a disease-causing point mutation or a neutral polymorphism (resulting in no apparent change in phenotype). SNP occurs in the human genome on average once every kilobase, and hence, it is the most frequent form of sequence variation among individuals. It is also the simplest, most common, and stable form of DNA polymorphism. The frequency, stability, and relatively even distribution of SNPs in the genome make them particularly valuable as genetic markers. By analyzing SNPs it may be possible to predict the genetic risk of a developing certain disease, diagnose a disease more accurately, or predict a therapeutic response to a drug. Over 2 million SNPs have already been found in the human genome, of which more than 60 000 have been identified in coding regions. Evaluating the influence of these variations on the activity of the enzymes involved in the metabolic pathways of the cell has important implications for pharmacology. Thus, effective analytical strategies for SNP detection will aid in the goal of developing a therapy based on an individual patient's genetic profile. Moreover, large-scale SNP analysis will provide new insight into evolution in biology as well as the history of human populations. In order to be both practical and cost effective, such complex genetic diagnostic approaches will require high-throughput analytical methods amenable to automation. Further details and a more thorough introduction into the principles of genetics and genetic analysis can be found in the literature cited.[1,2]

Capillary electrophoresis (CE) is an analytical method with low operation costs; it is suitable for high-throughput analyses due to its high-speed separation capabilities and the potential to be fully automated. These clear advantages have been responsible for the increasing range of applications of CE in clinical diagnostics over the past decade. In this paper, we present a comprehensive review of the methodological and instrumental development of CE systems toward ultrafast and sensitive DNA diagnostic techniques as well as a survey of the related principles and applications. This review is aimed at (i) the experts in DNA diagnostics by providing a useful reference on the current status of the relevant CE methodology and its future potential in the field, (ii) CE experts as a reference on current applications in DNA diagnostics, and (iii) newcomers in both fields as well as the broader chemical community as a useful overview and comprehensive literature survey. We also include a brief outline of the principles and features of CE essential for applications in the field of DNA analysis, in particular, the issues of separation selectivity and sensitivity. More general applications can be found in some comprehensive overviews.[3−11] CE applications in DNA analysis are also addressed in several monographs[12−15] and review articles.[16−24]

Extensive utilization of DNA diagnostics including sequencing has stimulated development of other high-throughput and cost-effective techniques as alternatives to CE.[25−28] Among those methods which have already proved their potential in this field are hybridization on chemical chips (or DNA microarrays),[29−31] in situ hybridization,[32] denaturing

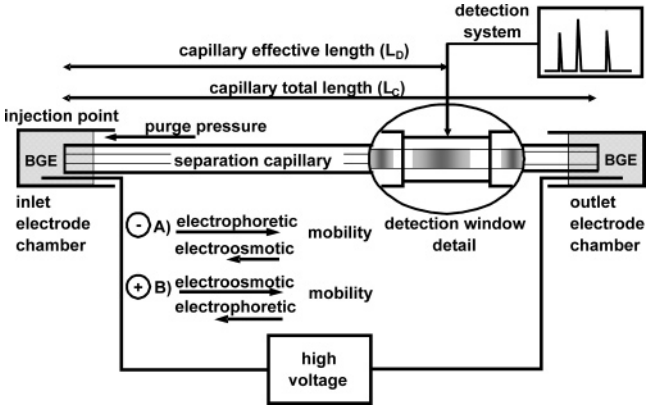


Figure 1. Basic CE instrumentation. (A) Negative electrode at the injection end—EOF slows down electrophoresis of anions. (B) Positive electrode at the injection end—EOF also carries anions to the detector in the reverse order to their electrophoretic mobilities—the higher the mobility of an analyte, the longer its migration time.

liquid chromatography,[33−39] mass spectrometry,[40] flow cytometry,[41] quantitative or real-time polymerase chain reaction (PCR),[42] pyrosequencing,[43−47] and single-molecule sequencing.[47−55]

2. Capillary Electrophoresis

The most successful development of an analytical tool for high-throughput, cost-effective, and reliable DNA analysis is in the area of miniaturized CE systems[16,17,24,56−67] including electrophoresis on micromachined microfluidic devices (or chips).[68−89] The most important advantage of CE, when compared to other liquid-phase separation techniques, is the analysis speed combined with the extraordinarily high separation efficiency.[90−93] In narrow capillaries with high electric resistance and high capacity to dissipate Joule heating, electric field strengths as high as 1 kV/cm can be used. Consequently, the analysis time is typically 1 order of magnitude shorter than that found in conventional slab gel electrophoresis (SGE). Another remarkable feature of CE is that the operational conditions can be kept constant, defined, and reproducible for weeks by filling the capillary with fresh electrolytes prior to each analysis. The analytical potential of CE has been proven by the massive implementation in the Human Genome Project (HGP) in which about 3 billion base pairs (bp) of human DNA were sequenced de novo utilizing capillary sequencers.

2.1. Basic Principles

The basic CE instrument setup is presented in Figure 1. The key element is the separation capillary. At present, separation capillaries are made of fused silica with outer surfaces coated by a polyimide to protect the fragile material during manipulation. The most common dimensions are as follows: the inner (i.d.) and outer diameters (o.d.) range from 10 to 100 and 100−360 μm, respectively; the thickness of the protective polyimide layer is approximately 15 μm; the effective (L_D) or total (L_C) lengths are usually between 20 and 60 cm. The applied voltage is generated by a high-voltage power supply; usually it ranges from 2 to 30 kV and once selected is stabilized. The electric current through the capillary typically lies between 2 and 50 μA, and it is obviously dependent on the applied voltage and electrolytic conductivity of the background electrolyte (BGE) used.

The detection system consists of a detector as well as the data acquisition and evaluation system. The most frequently used detectors are spectrophotometers or fluorimeters working in the UV or visible region and mass spectrometers. The detection window for the optical detector, which is made by removing the protective layer from the capillary, is usually distanced by a few centimeters from the capillary outlet (see the detail in Figure 1).

2.1.1. Electrophoretic Mobility and Separation Selectivity

When a voltage is applied to an electrophoretic system the ions of the analyte start to migrate. Their migration velocity depends on their properties in the BGE and the applied voltage, i.e., on the electric field strength E. The electric field strength is a ratio of applied voltage U and the total capillary length L_C ($E = U/L_C$). By relating the velocity v to the unit electric field strength E we derive the electrophoretic mobility μ

$$\mu = \frac{v}{E} \tag{1}$$

The electrophoretic mobility is the velocity of an ion in an electric field of 1 V/m and considered to be characteristic for an analyte in a separation medium of a given chemical composition, pH, ionic strength, temperature, etc. It is independent of the applied voltage and capillary used and, therefore, can be considered to be a qualitative parameter, i.e., a parameter serving for identification of separated substances.

The mobility can be evaluated from an electropherogram using a migration time t_m of an analyte zone migrating the distance L_D from the injection point to the detector at a voltage U applied across a capillary length L_C

$$\mu_{app} = \frac{L_D L_C}{t_m U} \tag{2}$$

The subscript app indicates that it refers to the apparent mobility since a bulk movement of the liquid inside the capillary may be present due to electroosmotic flow (EOF). This kind of convective mass flow is an inherent feature of electrophoresis. In CE it is a bulk solution movement with respect to the inner charged surface of the capillary.

Since the silica capillary wall is negatively charged due to the ionized silanol groups, the induced net charge of the solution inside the capillary is positive; therefore, the EOF is cathodic. The movement of the positive uncompensated charges in the electric field toward the cathode is spread due to the viscous forces through the bulk liquid inside the capillary. The mobility of the electroosmotic flow μ_{EOF} can be defined similarly to that outlined in eq 1. The net effective electrophoretic mobility can be evaluated from the apparent mobility μ_{app} as follows

$$\mu = \mu_{app} - \mu_{EOF} \tag{3}$$

If an analyte is neutral and moves only due to EOF, then its migration time is t_{EOF}, the effective electrophoretic mobility μ is zero, and its apparent mobility μ_{app} is equal to μ_{EOF}. Such an analyte can be used as an EOF marker. It follows then that μ_{EOF} can be evaluated by substitution of t_m by t_{EOF} in eq 1. It should be kept in mind that mobilities are signed quantities. Cathodic electromigration of an analyte is des-

ignated as positive (+) along with the mobility (scheme B in Figure 1). Anodic electromigration and mobility are negative (−) (scheme A in Figure 1).

The power to separate two similar ionic species by capillary electrophoresis is quantitatively described by the separation selectivity *S*. This is defined as the relative difference in velocities between two analytes migrating in the same direction (either cathodic or anodic).

Using effective mobilities the selectivity is expressed as

$$S = \frac{\Delta\mu}{\mu_{av} + \mu_{EOF}} \quad (4)$$

where μ_{av} is the average of the effective mobilities. For closely migrating analytes, the average of the effective mobilities may be replaced by the effective mobility of one of them.[94]

As mentioned earlier, mobilities are characteristic for individual species and may be used for analyte identification. In current practice, however, the most practical qualitative parameter is the migration time t_m. Of course, the prerequisite is that the same voltage is always applied across the same capillary and the capillary exhibits constant EOF. Moreover, the samples should be of similar composition, and it is especially important that the ionic strength of the analyzed sample should remain constant. If not, the migration time of a given analyte would vary and its value as an aid for identification would be lost.

2.1.2. Concentration Effects

Obviously, ionic species of the sample may contribute significantly to the electric conductivity of their own zones. Thus, the local electrolytic conductivities of zones vary with their composition and concentration. An inherent feature of CE is that the electric conductivity at a capillary cross section determines the electric field strength, and therefore, the migration velocity of the ions may vary both with position and time.[3] This results in important phenomena such as sample accumulation or dilution. These effects are especially pronounced at the point of sample injection. To predict the behavior of the system, some fundamental principles must be considered. The most important one is the Kohlrausch regulation function (KRF). This function expresses the fact that the electrophoretic processes are regulated by the initial conditions of the system. Its numerical value ω is locally invariant with time and defined as

$$\omega' = \sum_i \frac{c_i}{|\mu_i|} \quad (5)$$

provided that only systems with fully ionized monovalent ionic species are involved.[95] c_i and μ_i refer, respectively, to the concentrations and actual mobilities of all ionic species. For weak uni-univalent electrolytes, the specific derivation of KRF can be found elsewhere.[96] The governing principle can be explained as follows: when a sample with a KRF lower than that of BGE is injected, the analytes are then concentrated by crossing the original boundary between the sample and BGE zones. Since the conservation of mass obviously applies to the analytes this results in the sample zone becoming shorter in a capillary of constant inner cross section. This process is called migrating electrolyte sample stacking and a direct consequence of the concentration adjustment according to KRF.

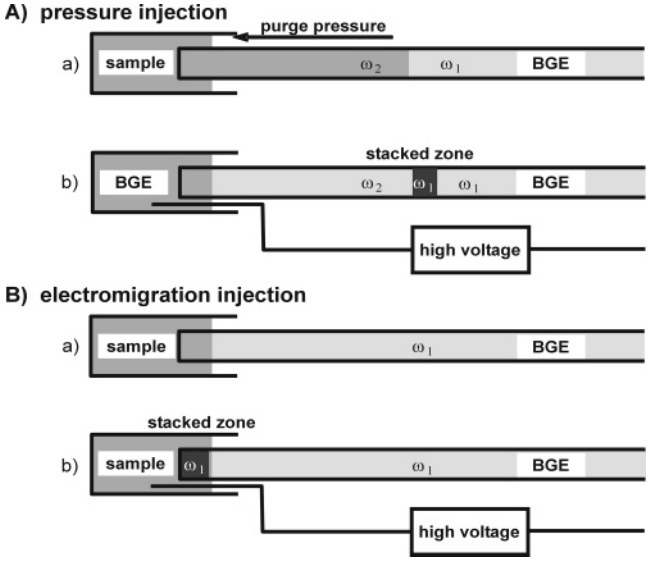


Figure 2. Scheme of sample stacking during (A) pressure and (B) electromigration injections. Kohlrausch regulation functions: BGE = ω_1, hydrodynamically injected sample zone = ω_2.

Figure 2A represents sample stacking in the case of hydrodynamic or pressure sampling, where the plug of a low-concentration sample is introduced by overpressure applied in a vial with the sample. Panel a indicates that the zone of a sample with a KRF value of ω_2 is introduced behind BGE with a KRF of ω_1. In classical commercial instruments, the zone is several millimeters long. Then, the injection end of the capillary is dipped into a vial with BGE and the voltage is applied (panel b). The sample zone is stacked so as to reach a KRF of ω_1. It should be noted that the BGE behind the boundary between zones ω_1 and ω_2 will have a low concentration resulting from its adjustment to a KRF value of ω_2 as in the original sample.

Figure 2B depicts stacking during electrokinetic sample injection. In panel a, the capillary filled with BGE with a KRF of ω_1 is placed in the vial with a sample. Then, the voltage is applied (panel b), and the sample is stacked in order to reach a KRF of ω_1 inside the first few millimeters of the capillary. Then the voltage is interrupted, the capillary inlet is dipped into a vial with BGE, and analysis starts by applying the voltage again. Of course, stacking of the analyte is only possible if the sample is less concentrated than BGE ($\omega_1 > \omega_2$). If the sample has a higher KRF value, then it is diluted during electromigration.

Another aspect of sample stacking is related to both the stabilized and nonstabilized moving boundaries. If the mobility of an analyte is smaller than that of a co-ion of the BGE, then the front boundary of the sample zone is stabilized, i.e., it is in a steady state. This phenomenon is called the self-sharpening effect, i.e., electromigration acts against diffusion dispersion. Simultaneously, the rear boundary of the sample zone becomes more and more dispersed with time. In the opposite case, when the mobility of the analyte is higher than that of the co-ion of a BGE, the rear boundary of the sample zone is sharp and steady and the dispersion of the front boundary increases with time.

Obviously, it is best to stabilize both the front and the rear boundaries simultaneously using appropriate running electrolytes: a leading electrolyte in front of the sample and a terminating electrolyte behind it. This procedure is called isotachophoretic stacking. Using this method even trace components of a sample can be stacked effectively between

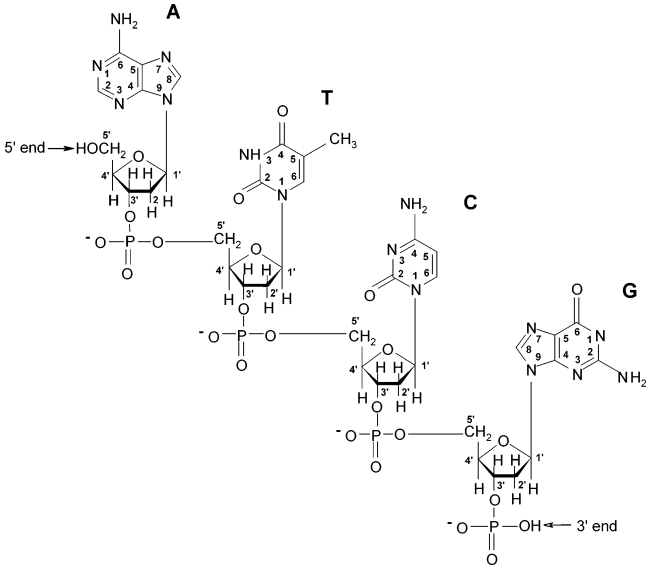


Figure 3. DNA primary structure: A = adenine, T = thymine, C = cytosine, G = guanine.

a terminator and a leader.[97] Various concentration techniques have been investigated with the aim of increasing both the sensitivity and the resolution of DNA analyses. By introducing the electrokinetic injection from relatively large volumes (several microliters) of carefully desalted samples, a more than 100-fold concentration can be achieved.[98−100] On the other hand, the presence of anions with high electrophoretic mobilities in the sample can positively affect its concentration during injection.[101,102] For example, chlorides or hydroxide anions, migrating in front of the lower mobility DNA during injection, can create a transient zone behind which the polynucleotide anions are stacked.[103−106] This technique is frequently called on-column transient isotachophoresis. It has also been found that a concentrated zone of sodium anions migrating in the opposite direction against DNA zones can improve their fronting in the regular tris-borate buffers.[107]

2.1.3. Electromigration of DNA

Size-based separation of homogeneous polyelectrolytes, e.g., DNA, SDS-denatured proteins, or other linear polymers with constant charge to size ratios, is not feasible in a continuum environment of free solutions of electrolytes. This is due to the proportionality of the friction hydrodynamic force and total charge of the molecule to its length. The friction hydrodynamic forces exerted on the relatively loose free-drained polymer coil while it moves as well as the accelerating electrostatic force both increase proportionally with addition of a nucleotide to the chain. This can be seen by considering the DNA primary structure shown in Figure 3.

Therefore, in order to induce a size-dependent effective electrophoretic mobility, molecular mechanical obstacles are dissolved in BGE. Such obstacles are called sieving media and can consist of physical or chemical gels as well as solutions of linear polymers. Once a sieving medium is present in the BGE, the migrating polyelectrolyte analytes are selectively retarded depending on their sizes and conformations.

In several papers the concept of end-labeled DNA fragments separated in free solution (ELFSE) was theoretically explained and experimentally confirmed.[108−114] The principle of this technique is that an uncharged polymer attached to

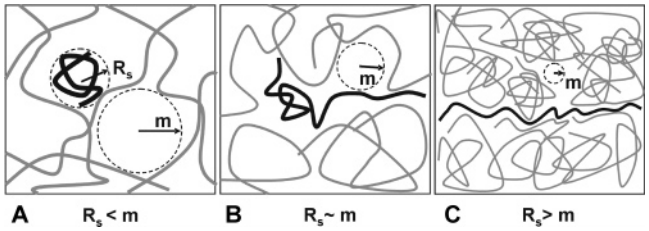


Figure 4. Schematic interpretation of DNA migration regimes: (A) Ogston sieving, (B) reptation, (C) reptation with permanent stretching. R_s = radius of hydrodynamic equivalent sphere; m = mean mesh size.

the polynucleotide molecule changes the constant ratio of the charge to the friction of a free-drained DNA polymer coil. Thus, the "hydrodynamic parachute" increases the friction force by a constant resulting in the differences in the free solution electrophoretic mobilities of modified DNA molecules being determined mainly by their charges. As a result, the end-labeled molecules with longer DNA segments migrate faster than the shorter ones. The potential of this technique has been demonstrated by the separation of DNA sequencing fragments[111] and detection of SNPs.[113]

The separation mechanisms of DNA in sieving media have been investigated by a number of approaches, including theoretical physical analyses,[115−124] computer simulations,[125,126] spectroscopic studies, and the direct monitoring of individual molecules by video microscopy.[115,127] Three migration regimes of a sample polyelectrolyte may be distinguished while it moves through sieving networks of various concentrations. Figure 4A shows a DNA polymer coil with the radius of hydrodynamic equivalent sphere, R_s, smaller than the average mesh size, m, of the network. In this scenario migration is controlled by the accidental interactions of the coil with the obstacles of the sieving medium. A rather different situation occurs when the R_s of the DNA polymer chain is comparable to the mesh size m (Figure 4B). In this case, the DNA molecular coil is occasionally elongated by squeezing through narrow pores and tends to expand in void regions. Sometimes it can get temporarily "hooked" and subsequently unravels like a rope sliding over a pulley. The velocity of such a migration is then strongly dependent on the length of the DNA chain. This mechanism of electromigration is called biased reptation. When the DNA molecule is much longer than the average mesh spacing (Figure 4C), a strong entanglement of the sample in the mesh may be expected and the molecule migrates in a "stretched" state nearly all the time.

A schematic dependence of the relative electrophoretic mobility of a polyelectrolyte on the logarithm of its molecular size M is presented in Figure 5. The dependence illustrates the typical variation of mobility for molecules which migrate through a sieving medium in the three regimes mentioned above. Its course is also determined by the concentration of the sieving medium and electric field strength. In region A the polymer coil can be considered to be a rigid sphere undergoing biased Brownian movement. The sample separation under these conditions is explained by the sieving model. The mobility of the sample is inversely proportional to the probability of its interaction with the fibers forming the sieving network. In this region, called the Ogston migration regime,[128] the spherical particle collides with the network at only one place at a time, i.e., the polymer chain is not entangled with the sieving matrix. Under this assumption the following relationship between the electrophoretic mobil-

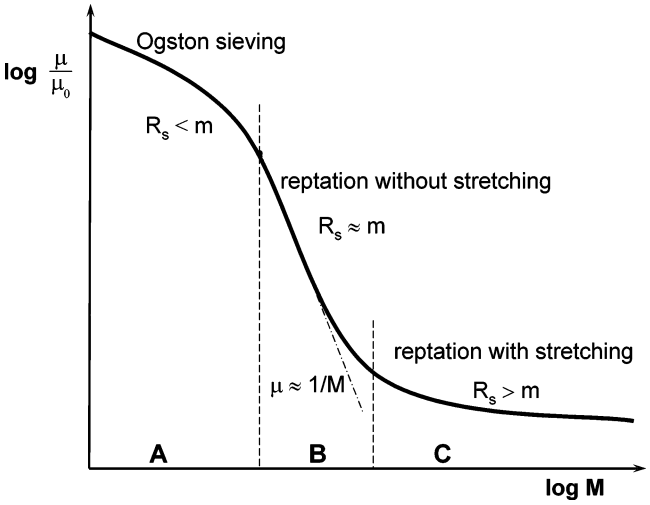


Figure 5. Schematic dependence of the logarithm of the relative mobility μ/μ_0 on the logarithm of molecular size M. Migration regimes: (A) Ogston sieving, (B) reptation, (C) reptation with permanent stretching. R_s = radius of hydrodynamic equivalent sphere; m = mean mesh size.

ity, μ, the sieving medium concentration, T, and the radius of hydrodynamic equivalent sphere of a DNA polymer coil, R_s, has been derived by Rodbard and Chrambach[129]

$$\mu = \mu_0 \exp[-kT(r + R_s)^2] \quad (6)$$

μ_0 is the electrophoretic mobility in a free electrolyte, r is the thickness of an obstacle fiber, and k is a proportionality constant. The most significant drop in electrophoretic mobility with the molecular mass of the sample is in regime B. In other words, the separation selectivity is the highest here. According to Lerman and Frisch's model[130] the mobility of a flexible molecule migrating by the reptation mechanism is inversely proportional to its size M

$$\mu \approx \frac{1}{M} \quad (7)$$

This relationship, however, is valid only if the electric field strength E is small. When E increases, another term, which is size independent and depends on E, must be added. This mechanism of so-called biased reptation with stretching was described by Lumpkin et al.[131]

$$\mu = \mu_0\left(\frac{1}{M} + bE^2\right) \quad (8)$$

Here, b is a function of the mesh size, m, of the polymer network, charge of the migrating polyelectrolyte, and temperature. From this equation it can be derived that the mobility of the migrating molecule tends to be size independent if either E or M is very high. The reason for this is a permanent elongation of the molecule dragged through the separation medium. As is clear from Figure 5, the tendency for the permanent stretching of a molecule increases with its length, but even short molecules can be permanently stretched if the electric field strength is high. The subsequent decrease in the separation selectivity is presented in Figure 5, region C.

2.2. Sieving Media for DNA Separations

The intensive development of sieving media and optimization of separation conditions over recent decades has been crucial for successful application of CE to the HGP. Employment of separation capillaries coated with permanent or dynamic coatings[132,133] and filled with replaceable sieving media allow a separation efficiency approaching 10 million height equivalents of the theoretical plates (HETP) to be reached. Several reviews following these developments have been published.[20,60,134−142] The choice of the suitable sieving media for CE was influenced mainly by more than one-half century's long experience with slab gel electrophoresis (SGE). The first gel electrophoresis experiments were most likely those performed by Tiselius in 1927, when he separated red phycoerythrin from blue phycocyanin in a slab of gelatin.[143] Probably the earliest report on the use of a hydrophilic support medium was published in 1950 by Gordon et al., who proposed 3% agar for the separation of proteins.[144]

2.2.1. Chemical and Physical Gels

Analogous to SGE, chemical or physical gels were the first sieving media used for separation of DNA fragments in capillaries.[145−149] The rigid structure of polymers in the gel state was frequently regarded as an unavoidable prerequisite for satisfactory resolution. Cross-linked polyacrylamide, prepared by an in situ radical copolymerization reaction of acrylamide with varying amounts of *N*,*N*′-methylenebisacrylamide (Bis), as cross-linker, catalyzed by *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TEMED) and ammonium peroxydisulphate (APS), was a widely used medium for separation of homogeneous biopolymers.[145−148,150] The monomer and cross-linker concentrations in mass percent are usually denoted as % T (mass percent of monomer in the solution prior to polymerization) and % C (mass percent of cross-linker with respect to the total amount of polymer), respectively. The usual concentration of polyacrylamide gels polymerized in capillaries was in the range 3−6% T and 5% C.

Alternative cross-linkers, e.g., ethylene diacrylate, polyethylene glycol diacrylate, *N*,*N*′-1,2-dihydroxyethylene bisacrylamide, *N*,*N*′-diallyltartardiamide, *N*,*N*′-bisacrylylcystamine, *N*,*N*′-bisacryl piperazine, and allyl-β-cyclodextrin, have been investigated with the aim of achieving not only optimal separation properties but also the highest possible order of reactivity for copolymerization with acrylamide.[140,151,152]

Similarly, various derivatives of acrylamide monomers, mostly mono- and disubstituted acrylamides, have been studied because of their high reactivity and in order to improve the physicochemical properties of the gels.[140] The acrylamido sugars, such as *N*-acryloyl-1-amino-1-deoxy-D-glucitol, produce highly hydrophilic polymers of high molecular mass allowing preparation of matrices of increased porosity.[153] Gels derived from acryloyl morpholine cross-linked with bisacryl piperazine and *N*,*N*′-dimethylacrylamide copolymerized with hydroxyethyl methacrylate (tradename HydroLink) are amphiphilic, i.e., compatible with a number of polar organic solvents.[154] The remarkable improvement in stability provides *N*-acryloylaminoethoxyethanol (AAEE)[155] and *N*-acryloylaminopropanol (AAP)[156] gels with more than a 500-fold increase in resistance to hydrolysis when compared to conventional acrylamide gels. In addition, these gels show high hydrophilicity, which is essential for protein separations. The AAP monomer was developed as a medium without the tendency to autopolymerize, which is otherwise observed in stock solutions of AAEE.[157]

The acrylamide gels are separation media with small pore sizes, making them suitable for separation of proteins and

oligonucleotides. The pore sizes range from a few nanometers in concentrated gels (20% T, 5% C ≈ 1.2 nm) to up to tens of nanometers in diluted gels (5% T, 2.66% C ≈ 30 nm).[158] It has been shown, however, that gels with pores as large as 500 nm can be produced. This occurs when gelling proceeds at a low temperature or in the presence of another polymer (e.g., polyethylene glycol, polyvinyl pyrrolidone, or hydroxymethyl cellulose), thus forming so-called mix-bed matrices.[138,140,158,159] The effect responsible for the substantial increase in pore size is an interchain bundling of the growing polyacrylamide chains prior to the cross-linking reaction. Thus, pores are generated by competition between gelation and phase separation (called spinodal decomposition of the sol) similar to that found in agarose solutions. The size of the pores is determined by the stage at which the gelation process stops the decomposition. Whereas in agarose sols the pores are closed by the physical entanglement of the polysaccharide helices, in polyacrylamide-decomposed sols it is achieved by the chemical reaction of a cross-linker.[158] In such mixed-bed gels DNA fragments as large as 21 kbp can be separated.[158] Chiari et al. reported gels containing polyacrylamide covalently linked to agarose with an average pore size 30% higher than the value of a regular Bis-cross-linked gel of the same % T. Due to the agarose-induced gelation process during polymerization, the matrices are more elastic and mechanically stronger than classical polyacrylamide gels.[160]

The extent of incorporation of the various monomers into the polyacrylamide gels was an issue intensively studied in the early 1990s. In the conventional system with TEMED and APS as initiators the degree of conversion from monomer to polymer reaches approximately 90% within the first 15 min.[161] In the photopolymerization of polyacrylamide gels in the presence of methylene blue as photoinitiator together with a redox couple (sodium toluene sulfinate, a reducer, and diphenyl iodonium chloride, an oxidizer) very high conversion efficiencies (>96%) over the pH 3−10 range were attained. These gels produced by photopolymerization were considered the most suitable for gel electrophoresis.[162]

Preparation of gel-filled capillaries is not an easy task. Shrinkage of the gel and formation of bubbles during polymerization must be prevented. Several strategies, including polymerization under high pressure, polymerization with addition of a neutral linear polymer, or gradual polymerization along the capillary length, have been described in the literature (reviewed by Dolnik[134]). The fact that the gel cannot be replaced between analyses presents other issues. Any change in the chemical or physical structure of the gel inside the capillary affects the reproducibility of migration times. The most common reasons for degradation of gel capillaries are a low resistance of the gel to alkaline or acidic hydrolysis, formation of bubbles due to the Joule heating during electrophoresis, mechanical damage to the gel in flexible capillaries, and deterioration of the gel by impurities, e.g., by long fragments of DNA sequencing templates, sequencing enzymes, etc. However, the principal problem affecting the ability of a capillary to be reused is the consecutive increase of the resistance due to ion depletion at the capillary ends. Thus, gel capillaries are typically used only for three or four analyses.

Theoretical analysis of the formation of anomalous conductivity zones in gel electrophoresis as well as experimental verification of this phenomenon has been investigated by Spencer.[163−165] He showed that the main reason for this phenomenon is the change in mobilities of the electrolytes when migrating from free solution into a gel and vice versa. Such a change in mobility is likely to be a result of the collisions of the electrolytes with the polymer molecules of the matrix. When the mobilities of an ion and co-ion do not change by the same factor, i.e., their transference numbers differ, there will be unequal rates of transport of each ion at the two sides of the interface. Consequently, changes in ion composition are developed as described by Hittorf in his well-known experimental method for measuring transference numbers.[166] Thus, an ion is depleted on one side of a gel column, while its concentrated zone appears on the opposite side. Moreover, if the transference numbers are concentration dependent, one can expect the movement of such zones through the column[163] as well as a resulting change in pH.[164] This will, of course, affect not only the overall conductivity of the column but also migration of the solute zones. In practice, two procedures help to compensate for these effects: (i) periodical trimming of the ends of the capillaries to remove the ion-depleted zones before each analysis and (ii) performing electrophoreses with the capillary ends immersed in electrode chambers filled with the gel of the same composition as the one inside the capillary in order to adjust equal transference numbers.

The latest developments in polymer chemistry have opened up new possibilities in the preparation of cross-linked polyacrylamide gels. Recently, use of a photoinitiated polyacrylamide gel (ReproGel; Amersham Pharmacia Biotech, Piscataway, NJ) in a microfabricated device has been demonstrated.[167−169] By substituting UV-activated initiators for standard chemical initiators, relatively short polymerization times (typically 10 min) have been attained. Moreover, the electrophoresis gel can be precisely positioned at any location within a microfluidic network by selectively masking the device during UV curing. However, in spite of these advantages the application still suffers from the common drawbacks of gel electrophoresis as discussed above. Overall, the main reason for employing cross-linked polyacrylamide gels was to enhance the resolving power in microscale systems allowing for resolution of DNA sequencing fragments on compact microchips. Thus, separations of a standard single-stranded DNA (ssDNA) ladder with fragments differing by 20 bases as long as 400 bases and ssDNA fragments of 195, 200, and 205 bases were demonstrated for 8% ReproGel with migration distances of 1.5 and 1 cm, respectively. Enhanced size resolution on cross-linked high-concentration gels is also a prerequisite for application of very low voltages across short migration distances. This makes the idea of a portable battery-powered device realistic. The standard fragments were separated at an elevated temperature of 50 °C at an electric field strength of 16 V/cm in 15 min.[23] Cross-linked polyacrylamide was judged as the most versatile sieving matrix for separation of both ds- and ssDNA fragments (from oligomers to 1000 bases) over distances around 1 cm. A solution of 13% linear polyacrylamide (molecular mass not specified) was found to be a medium with less than optimal performance in microfabricated systems, especially at low electric field strengths.[169] Agaroses and various copolymers including Pluronic gels represent the most common of the physical gels used in capillaries. These substrates can be easily filled into a capillary and replaced after analysis in the state of sol.[170]

Besides the cross-linked polyacrylamide chemical gels described above, physical gels represented by a polysaccha-

ride agarose have been tested as a separation medium in capillaries. Agarose, a widely used large pore size medium in SGE, exhibits a marked thermal hysteresis in sol−gel and gel−sol transitions. The capillary is first filled with a solution of agarose at a temperature over its melting point, and then the sol is allowed to gel inside the capillary, which is cooled below the gelling temperature. After separation the medium is removed at a temperature increased to over the melting point. Both temperatures, which differ by several tens of degrees Celsius, are dependent on the chemical composition of the polysaccharide. Nowadays, there is a wide choice of modified low melting point agarose media suitable for CE applications. Schomburg's group demonstrated complete separation of DNA restriction fragments (ϕX−174, Hae III digest) in a 2% agarose gel at temperatures of 10, 15, and 25 °C. A low melting point Agarose Wide Range (Sigma) (mp 65 °C, gp 35 °C) medium at concentrations of 0.7−5% was replaced after each analysis at 90 °C.[149] Zhao et al. demonstrated a fast separation of PCR fragments in 3.5 cm long channel microfabricated from poly(dimethylsiloxane) and filled with agarose gel.[171]

2.2.2. Polymer Solutions

Implementation of polymer sols into the practice of CE has been the most efficient approach solving all the problems connected with gel-filled capillaries. These true polymer solutions provide relatively low viscosity media which can easily be replaced after each analysis, and thus, identical analytical conditions can be ensured for all consecutive separations. There are other advantages which favor polymer solutions to gels: (i) mechanical destruction of the sieving polymer by capillary coiling is avoided, and hence, wall coatings predominantly determine the life time of capillaries; (ii) samples can be introduced by either a hydrodynamic injection controlled by overpressure or a vacuum at the capillary ends; (iii) EOF induced in bare fused silica (noncoated) capillaries can be used as a nonselective transport, carrying DNA fragments to the detector against their electromigration, i.e., in an order inversely proportional to their sizes; (iv) both the concentration and type of sieving medium can be changed in consecutive runs; (v) selectivity of the separation in polymer solutions of optimized molecular mass is just as high as in polymer gels; (vi) polymer solutions facilitate automation of CE analyses.

To explain the separation of DNA fragments in the polymer sols, the concept of transient dynamic pores or tubes in the entangled polymer solutions has been adopted.[172] The dynamics of temporary transient states of a mesh of polymer fibers can be described by its relaxation time, a medium lifetime of the dynamic "pores". If the selective retardation of migrating molecules is expected, the contact time of these molecules with the mesh (determined by their mobilities and electric field strength) should be higher than the relaxation time. By comparing the characteristic relaxation times of entangled polyacrylamides ($\sim 5.9 \times 10^{-4}$ s), cross-linked polyacrylamide gels ($\sim 4 \times 10^{-3}$ s), and the contact time of DNA fragments ($\sim 1-8 \times 10^{-4}$ s) it was concluded that the sharp resolution of long DNA fragments needs the separation medium to consist of long molecules.[173] The fact that the dynamic pores in polymer solutions are not rigid obstacles for migration of polynucleotides was described as the constraint release migration mechanism. Thus, the long DNA molecules penetrate a sieving medium without a proper retardation. However, the constraint release can also play a positive role in the separation since it reduces the tendency to the permanent DNA stretching, a reason for zero selectivity in dense media and at high electric fields.[116]

In 1977, Bode demonstrated the sieving effect for polymer solutions of polyethylene glycol (PEG) and linear polyacrylamide (LPA) for separation of SDS-denatured proteins in glass cylindrical columns.[174,175] Since that time considerable effort has been spent optimizing the separation conditions and testing the ability of different polymer solutions to effectively separate both ss- and dsDNA fragments. In 1989, Zhu et al.[176] and Hjertén et al.[177] demonstrated the separation of DNA standards in capillaries filled with 0.5% solutions of hydroxypropylmethyl (HPMC) and methyl cellulose (MC).

Boček et al. demonstrated the separation of DNA standards ranging in size from 72 bp to 12 kbp in a liquefied low melting point agarose. The agarose sol was prepared at a temperature well above its melting point and subsequently cooled down to a separation temperature of 40 °C, which is still above the gelling temperature (28 °C).[178−180] In following publications the use of solutions of linear polyacrylamide[181,182] and liquefied agarose[183,184] in DNA separations using CE was described. The comparison of cross-linked (3% T, 5% C) and linear (9% T) polyacrylamides revealed that the same separation selectivity can be achieved in both cases. However, in the case of the linear polymer this is at the expense of higher concentration and, hence, results in longer analysis times. The LPA proved to be capable of resolving a broader range of fragment sizes.[185]

In addition to the low melting point agaroses, some other polysaccharides, such as glucomannan[186] and galactomannans,[187] were investigated for their potential as efficient sieving media. Numerous derivatives of cellulose, hydroxyethyl (HEC),[188−205] hydroxylpropyl (HPC),[127,199,206−209] hydroxylpropylmethyl (HPMC),[102,176,177,198,210−217] and methyl (MC)[218−222] celluloses, were also investigated. These celluloses as well as other polysaccharides are frequently used as sieving media for ultrafast separations of DNA fragments in microfluidic glass and plastic devices because of their good separation and coating properties.[194,201,204,205,209,215−217,223,224] The polysaccharides were also successfully used for separation of RNA[212,213,225] and ssDNA[225−227] fragments. Barron et al. compared the separation properties of dilute and semidilute solutions of LPA, HEC, and HPC polysaccharides of several different average molecular masses for DNA fragments ranging from 72 bp to 23 kbp.[199] They demonstrated the superior resolution of DNA fragments in 0.2% solutions of HEC compared to PAA and HPC of the same concentrations. The reason may be that HEC shows higher hydrophilicity and stiffer molecules with extended conformations. The molecular stiffness is described as the persistence length of a polymer and can be used as a parameter characterizing the sieving properties of polymer solutions. Persistence length is a statistical quantity characterizing the mean length of a part of the random chain which "persists" as a straight line. The chain stiffness of hydrophilic HEC (persistence length $\approx$ 8.3 nm) is much higher than the chain stiffness of more flexible polymers such as LPA $\approx$ 1.73 nm, poly(dimethylacrylamide) (PDMA) $\approx$ 1.1 nm, or poly(ethylene oxide) (PEO) $\approx$ 0.65 nm.[192,228] It follows that the stiffer polymers adopt more relaxed conformations and, therefore, are entangled and provide a reasonable resolution at lower concentrations and/or molecular mass.[199]

The effect of molecular stiffness was also demonstrated by the successful separations of long DNA fragments in

ultradilute (<0.002% w/w) solutions of LPA[199] and HEC.[192,229,230] It has been shown that an extended conformation of stiff molecules increases the molecular retardation of DNA fragments as well as their separation selectivity.[199] Since the concept of dynamic pores is unacceptable in such media where their concentrations are far below the entanglement threshold, the principle of the size separations was postulated as causing DNA transient hooking and subsequent dragging of the uncharged polymer chains during migration. This migration mechanism was confirmed by videomicroscopy[231] as well as by a theoretical model.[119] It is obvious that stiffer polymers exert a higher friction force on the migrating DNA−polymer complex than more flexible chains.

Among the synthetic polymers used in CE, the linear polyacrylamide[232−235] and its derivative poly(*N*,*N*-dimethylacrylamide) (PDMA)[233,236−239] are by far the most frequently used replaceable sieving media for separation of both ss- and dsDNA fragments. Other derivatives, based on the mono- and disubstituted amido nitrogen of the acrylamide, were synthesized with the aim to (i) improve their resistance to alkaline hydrolysis, (ii) increase their hydrophilicity, (iii) optimize the porosity of the final polymer, (iv) enhance the self-coating ability, and (v) reduce the viscosity of the polymer solutions.[135,198,240−245] The resistance of LPA molecules to alkaline hydrolysis is low. At alkaline pHs the amide bonds are partly hydrolyzed and negatively charged carboxylic groups are formed on the polyacrylic chain. Experimental results showed that the degree of hydrolysis of LPA reached 5% at 50 °C and pH 12.5 in 1 h. A rapid onset of hydrolysis was observed at a temperature of 70 °C and pH 13. Under these conditions 17% of the LPA amide groups hydrolyzed after 1 h.[246] Various modifications of polymers based on the polyacrylamide chain have been designed to increase the stability. Righetti's group showed that mono- and disubstituted polyacrylamides are substantially more resistant to alkaline and acidic hydrolyses than the corresponding unsubstituted species.[155−157,247] For example, poly(*N*-acryloyl-2-amino-2-hydroxy methyl-1,3-propane) exhibited a 2-fold and poly(*N*,*N*-dimethylacrylamide) an even higher 500-fold resistance to alkaline hydrolysis than LPA.[155,247] The greatest resistance to hydrolysis was found in solutions of poly(*N*-acryloyl aminoethoxy ethanol) and poly(*N*-acryloyl aminopropanol) with only 1.22% and 1.1% of hydrolyzed amido bonds after 60 h treatment by 0.1 M NaOH at 70 °C.[156] It was found that the presence of substituents on the amido nitrogen in poly(acryloylaminoethoxy)ethyl-β-D-glucopyranoside reduced the viscosity of the polymer solution without changing the separation selectivity. In addition, an increase in the monomer size led to formation of a network with larger pore sizes.[242]

Investigation of some other synthetic polymers revealed interesting separation properties. Poly(ethylene oxide) (PEO) has been successfully used at concentrations from 1% to 3.5% for the separation of ds- and ssDNA fragments by several research groups. PEO, which is commercially available in a broad range of molecular masses (100 kDa to 8 MDa) in powder form (Aldrich, Milwaukee, WI), provides some valuable features such as easy preparation of homogeneous solutions, self-coating ability,[248] and relatively low viscosity when compared to solutions of LPA.[249] PEO together with poly(ethylene glycol) and poly(propylene oxide) are the components of the triblock copolymer Pluronic polyol F127, an effective separation medium used in CE for separation of biomacromolecules such as DNA fragments and proteins.[250,251] Yeung's group used PEO as a sieving matrix in a denaturing medium for high-speed DNA sequencing.[252−254] Separation of ssDNA fragments up to 1000 nts was demonstrated in a 3% PEO solution.[253] The same resolution was obtained using pulsed field capillary electrophoresis for DNA sequencing.[255] At a temperature gradient up to 65 °C or at a constant temperature of 65 °C (40 °C optimum), separation of ssDNA sequencing fragments was feasible even in a nondenaturing PEO solution.[256,257] A PEO solution was also used in the integrated on-line system coupling: a microreactor for a dye-labeled terminator cycle-sequencing reaction, subsequent sample purification in a size-exclusion chromatographic column, and CE separation of the sequencing fragments.[258] PEO solutions are also compatible with 96-capillary DNA sequencing systems.[259]

Application of PEO to genetic typing was demonstrated by observation of VNTR polymorphism in the human D1S80 locus. Here, a pooled allelic ladder, which contained the 27 most common human alleles, was used as the absolute standard. Repeated separation runs of dsDNA were performed in the same uncoated fused silica capillary.[260] Chang's group applied EOF to drive a counter-flow of PEO solutions through the capillaries. They showed several model separations including sweeping concentration of samples to prove the advantages of this arrangement.[208,261−265] The same group used this strategy to diagnose β-thalassemia through separation of PCR products (330 and 334 bp) taken from both a healthy person and an affected patient. Separation was accomplished within 15 min using a 1.5% PEO solution containing 2 M urea at 30 °C.[266] Xu et al. used a 1.3% solution of HPMC to separate dsDNA fragments 50−590 bp and mixed solution of PEO at a total concentration of 0.1% to separate fragments 520 bp to 20 kbp on a plastic chip.[216] Similarly, Madabhushi et al. used a mixture of a 0.5% solution of PEO (1 MDa) to separate dsDNA fragments up to 1.5 kbp, combined with 0.1% PEO (8 MDa) to separate fragments between 1 and 40 kbp, at 25 °C within a single run without the use of pulsed fields.[267]

Poly(vinyl pyrrolidone) (PVP) is a commercially available polymer with good sieving properties, low viscosity at moderate concentrations ($\sim$ 5%), and excellent self-coating properties, which may reduce EOF to a negligible level.[259,268] Yeung's group tested solutions of PVP (molecular mass 1 MDa) for DNA genotyping, mutation detection, and sequencing in individual uncoated capillaries and capillary array electrophoresis systems (CAE).[268−270] They successfully separated dsDNA fragments amplified on the human D1S80 locus (exhibiting VNTR polymorphism) and on amelogenin (a sex determination protein) as model samples. The STR in vWF, TH01, TPOX, and CSF1PO loci as well as the sequencing fragments of the M13mp18 plasmid were separated in the form of ssDNA up to 500 bases with a good resolution.[268] Munro et al. recognized the self-coating properties of PVP applied as a sieving medium in microchannels for chip-based assays of T- and B-cell lymphoproliferative disorders.[271] Although HEC provided adequate separation power for the separation of PCR-amplified fragments from the variable region of the T-cell receptor-gamma gene (150−250 bp range) and the immunoglobulin heavy chain gene (80−140 bp range) the additional coating step was necessary. PVP seemed to provide an adequate separation matrix without the need for the coating.[271] Good surface dynamic coating properties of PVP in combination with other coatings such as allyldimethylchlorosilane, 4-chlorobutyldimethyl-

chlorosilane, (γ-methacryloxypropyl)trimethoxysilane, chlorodimethyloctylsilane, and 7-octenyltrimethoxysilane in fused silica capillaries as well as in glass chips have been found.[204,272] PVP has been used as a promising sieving medium in capillaries[273,274] as well as in microfluidic separation systems.[275−279] An interpenetrating polymer network consisting of PDMA and PVP, synthesized by polymerizing *N*,*N*-dimethylacrylamide monomer directly in a PVP buffer solution, was successfully tested as a separation medium for dsDNA fragments.[280]

Poly(vinyl alcohol) (PVA) is mainly used as a coating agent to reduce EOF and adsorption of analytes.[281,282] However, its application as a sieving agent is also described.[183,283] Its good sieving properties are diminished due to strong tendency to self-aggregation both in solution and even more pronounced at the silica wall. Very strong hydrogen bonding is the reason for the relatively fast formation of physical gel in PVA solutions.

Poly(ethylene glycol) (PEG) sols show relatively high viscosity. Therefore, a modified PEG, end-capped with micelle-forming fluorocarbon tails, was designed for DNA sequencing. This polymer self-assembles in water into an equilibrium network of micelles with a well-defined mesh size. The strong non-Newtonian rheological properties of this gel-like structure are characterized by the viscosity decrease under shear, which is advantageous for replacing the medium after sequencing analysis. Under optimum conditions the resolution limit was 450 nts at 200 V/cm.[284]

Theoretically, for the successful separation of DNA molecules, their physical contact with a sieving medium is essential and sufficient. In practice, however, the situation is more complex and various enthalpic interactions affect migration of the polynucleotide chain. Not only the chemical interactions of DNA with the sieving media and BGEs[285,286] but also interactions of BGEs with the sieving media[215,287] play an important role in some instances. While the transient chemical interaction of a polymer analyte with a sieving medium can reduce the separation efficiency due to the slow kinetics, the existence of complexes of some BGE components with the medium improves the sieving properties and separation resolution.[287] Formation of complexes between borates and polyhydroxy polymers such as agarose and derivatives of cellulose can improve the entanglement of their diluted solutions. In this case, tetrahydroxyborate anions $B(OH)_4^-$ act as a central linkage and form a cross-linked polymer network of borate−diol complexes. Low molecular mass polyhydroxy additives such as mannitol, glucose, or glycerol in HPMC solutions with or without TBE buffer (1 × TBE buffer, pH 8.3, consists of 89 mM Tris, 89 mM boric acid, and 2.5 mM Na_2EDTA) also provide more selective DNA separations.[215,287,288] Formation of hydrogen bonds with the HPMC matrix and/or DNA probably increases the coupling interactions between the matrix and DNA molecules. As a result, very fast separations can be performed in easily replaceable, relatively low viscous, low molecular mass HPMC (11.5 kDa) in a microfluidic device with a migration path of 3 cm.[215] On the other side, formation of borate−agarose complexes, which increase the net negative charge of the agarose gel fibers, is responsible for the increased EOF in agarose gels prepared in TBE buffer.[285]

The effect of temperature on the separation selectivity and efficiency in CE of DNA fragments has been investigated by several groups.[184,289−293] Temperature affects the behavior of both the DNA fragments and the sieving medium. Therefore, although an increase in the capillary temperature can lead to an improvement in the resolution of one system it leads to deterioration in the other.[292,294] The theoretical model of biased reptation (eq 9) predicts that the thermal energy of a DNA molecule protects its permanent orientation and, consequently, the separation selectivity of long fragments will be improved at a higher temperature.[290,292] On the other hand, the dynamic character of the polymer medium and the temporary transient mesh network of polymer fibers should also affect selectivity and band broadening. A considerable decrease in the lifetime of transient "pores" at elevated temperatures will lead to a loss in separation selectivity. Thus, the optimum temperature must be found for a particular system.

In recent years, various alternatives to polymer solutions have been reported. Aqueous solutions of monomeric nonionic surfactants such as the *n*-alkyl polyoxyethylene ethers have been shown to be effective sieving matrices for the separation of DNA fragments. These surfactants self-assemble into dynamic long chains in solution and behave as dynamic polymers. Fast separation of dsDNA fragments ranging from 10 bp to 5 kbp and DNA sequencing fragments up to 600 nts were achieved in bare fused silica capillaries.[295] Mechanical obstacles less than micrometer size (prepared by a lithography technique in a separation channel) or suspensions of nanomaterials are other examples of alternative separation strategies.[296−298] Recently, several new approaches have been published: (i) entropy-based separations in an array of narrow and broad channel segments,[299] (ii) arrays of pillar-like obstacles,[300,301] (iii) randomly distributed nanospheres or magnetic self-assembling structures,[302,303] and (iv) small adhesion surface segments. Incorporation of nanostructures and nanomaterials into the practice of DNA analytical separations has recently been reviewed by Lin et al.[304]

2.2.3. Sieving Media for DNA Sequencing

Polyacrylamide gels were initially tested for the CE separation of DNA sequencing fragments. The separations were demonstrated to be three times faster with better resolution and higher separation efficiency than conventional automated slab gel DNA sequencing instruments.[146,147,305−311] The low stability and limited lifetime of the gel-filled capillaries were the main obstacles for the development of fully automated instrumentation with the capability to control many parallel analyses. Thus, development of replaceable sieving media was especially important for developing high-throughput DNA sequencing technology. Intensive research in this area showed that highly entangled solutions of hydrophilic, high molar mass polymers are required in order to achieve a high separation efficiency as well as a long read of DNA sequence.[312,313] Karger's group was the first to use replaceable LPA as a sieving matrix for DNA sequencing. The sequence of 350 nucleotides of phage M13mp18 was determined in 6% LPA in 30 min.[314] Dovichi's group also discovered various separation conditions for the application of replaceable LPA.[315−317] The relatively short read length was improved using capillaries filled with a replaceable LPA matrix operated at elevated column temperatures of up to 60 °C. As a result, Kleparnik et al. demonstrated the separation of DNA sequencing fragments extended to lengths greater than 800 bases.[292,318] An elevated temperature not only increased the sequence read length and analysis speed but also enhanced the denaturing ability of the separation

environment.[290,319,320] By combining an elevated temperature with the optimum composition of low and high molecular mass LPA and a sample cleanup from the DNA template, a considerable increase in the sequence read length up to 1300 nts was achieved.[312,321−325] A long read length, due to high separation selectivity and efficiency, is very useful especially for large-scale sequencing projects. It reduces the number of analyses and, thereby, the number of reactions and amount of reagents needed.

A low-viscosity solution of PDMA, developed for separation of DNA sequencing fragments,[232] became the sieving polymer in commercially available media POP-4 and POP-6 (PE Applied Biosystems, Foster City, CA). Both polymers dynamically coat the capillary surface, and therefore, bare fused silica capillaries can be used. Replaceable sieving matrices based on long-chain LPA require coated capillaries. The best results were obtained with the PVA covalently coated capillaries.[292,326] As already been mentioned, some other linear polymers HEC,[226,227] PEO,[252] and PVP[327] have been used for separation of ssDNA sequencing fragments.

Thermo-responsive and shear-responsive polymer solutions with "switchable" viscosities have been proposed for applications as DNA sequencing matrices for capillary and microfluidic device electrophoresis.[328] The shear-responsive (non-Newtonian) polymer matrices exhibit a rapid drop in viscosity as the applied shear force is increased. The thermo-responsive polymers display either a lowered or a raised viscosity as the temperature of the solution is elevated. These properties are attained by incorporation of moderately hydrophobic groups in some part of the polymer structure. These are responsible for either phase separation or hydrophobic aggregation at elevated temperatures. Such solutions with switchable viscosities can be rapidly loaded into separation channels under a low applied pressure.

Due to the extremely high intrinsic separation efficiency of DNA fragments, the effective migration distance can be reduced to several centimeters or even millimeters and the migration time reduced to minutes or seconds, respectively.[105,329−334] The limits of ultrafast separations of DNA sequencing fragments in LPA solutions were investigated in capillaries or microfluidic devices with effective migration distances as short as several centimeters. Muller et al. resolved 300 ssDNA sequencing fragments in a capillary of an effective length of 3 cm within 3 min,[332] and Woolley at al. used a microfluidic device of 3.5 cm effective separation distance to separate 433 bases in 10 min.[335]

2.3. CE Instrumentation for DNA Diagnostics

During the past two decades the vast technological advances in CE have opened up a range of new applications in the analysis of biopolymers as well as new horizons in clinical chemistry and molecular biology. The fact that total separation volumes lower than 1 μL and sample volumes as low as several tenths of nanoliters are feasible together with highly sensitive laser-induced fluorescence (LIF) detection has made the analysis of a single-cell content both accessible and convenient.[334,336−343] Detection of a single DNA molecule is now a reality.[344−353] CE instrumentation is also amenable to the on-line automated high-throughput fraction collection of DNA fragments after leaving the separation capillary. Several instrumentation strategies of preparative CE have been described in the literature: (i) direct discontinuous collection into the multiple vials,[148,354−358] (ii) continuous collection on a moving surface,[359] (iii) continuous collection into changing vials via a sheath flow liquid around the separation capillary exit,[360−364] and (iv) continuous comprehensive collection from multiple capillaries into a moving array of microvials formed directly in the slab of a conductive agarose gel.[365−367]

2.3.1. Fluorescent Labeling

Since the quantum yield of intrinsic fluorescence of the nucleotides is poor and requires deep UV excitation, labeling with covalently attached tags or fluorescent noncovalent staining dyes is necessary for sensitive detection. Dyes suitable for use as covalently attached labels for LIF of DNA must fulfill several criteria: (i) the absorption and emission maxima of an individual label should be as far from each other as possible in order to minimize the background due to the laser light scattering; (ii) the emission maxima of the applied dyes should be well resolved, allowing clear identification; (iii) the dyes should have high molar absorbances and quantum yields to provide sufficient detection sensitivity; (iv) they should not significantly affect the annealing of the primers or incorporation of labeled terminators during the polymerase reaction; (v) the electrophoretic mobility of the sequencing fragments should not be affected significantly. The effect of fluorophor labels on migration of ssDNA fragments has been reported in several papers, and it is worth mentioning that different labels may influence mobilities to different extents.[368,369] The most frequently used fluorophores with emissions in the visible region are derivatives of fluorescein, rhodamine, Texas red, NBD, BODIPY, and cyanine dyes. The structures of these dyes with some reactive groups for their attachment to the primers and dideoxynucleoside triphosphates are presented in Figure 6. Cyanine dyes belong to a class of dyes containing one or more methine (−CH═) groups linking two nitrogen-containing heterocyclic rings: $R^1N[-CH{=}CH]_nCH{=}NR^2$. There are three major classes of commonly used reagents for the labeling of amines: succinimidyl esters (including sulfosuccinimidyl esters), isothiocyanates, and sulfonyl chlorides. Such amine-reactive reagents are conjugated with nonprotonated aliphatic amine groups, e.g., with 5′ terminal amino-modified oligonucleotides.[370]

Fluorescent dyes for noncovalent DNA labeling are planar monomeric or homodimeric molecules that are capable of inserting (intercalating) between the neighboring bases in a ds- or ssDNA molecule. This kind of complexation usually changes the conformation of the DNA molecules and increases both migration time and separation selectivity. Such dyes are incorporated at a rate of 1 dye molecule for every 4−10 bp in dsDNA, and therefore, the fluorescence signal increases with molecular size. This is similar to what is found in absorbance detectors. Moreover, even a slightly changed conformation of the intercalated molecule results in a greater than 1000-fold fluorescence enhancement.[371] While the detection limits of the dyes themselves are in the zeptomolar range (10^{-21} mol), the detection limits of fluorescently labeled DNA molecules can even reach a few yoctomoles (10^{-24} mol).[372] In practice, the staining agent is directly added to the sieving medium and/or mixed with the DNA samples prior to analysis. Although noncovalent labeling is unselective, its simplicity and ease of use makes it convenient also in automated CAE systems.[373,374] The most commonly used intercalating dye in CE is ethidium bromide,[260,375−380] which has the ability to bind to both ss- and dsDNA, ethidium homodimer,[195,381−383] thiazole orange,[195,382,384,385] and oxazole

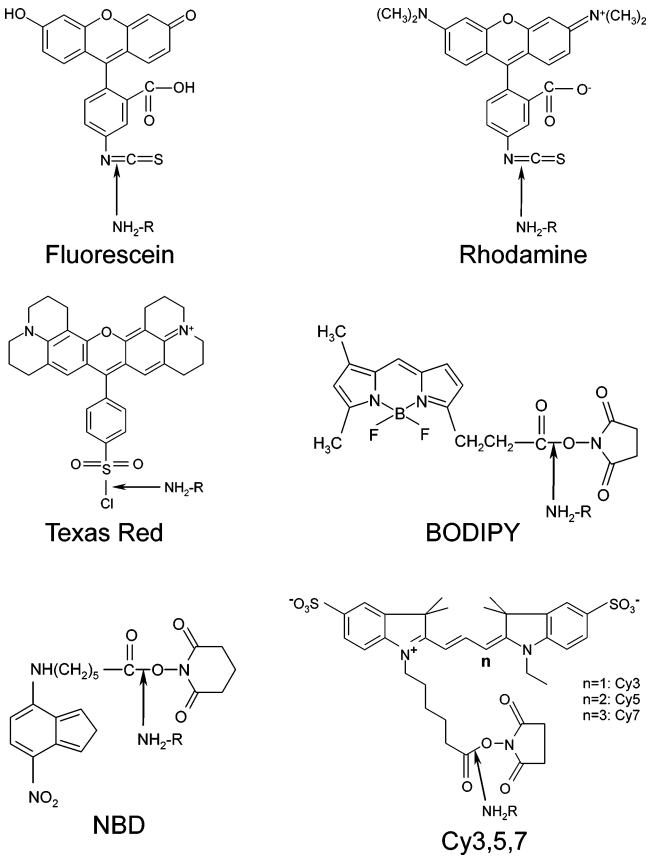
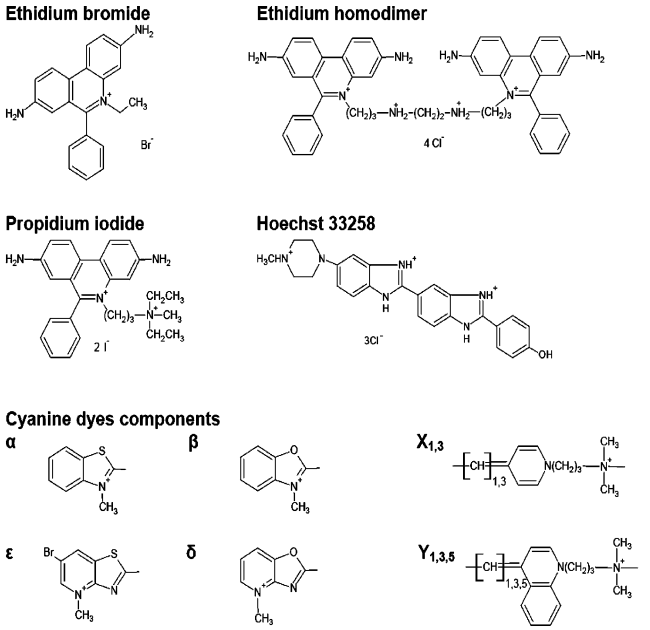


Figure 6. Some amine-reactive dyes frequently used for DNA labeling. Derivatives of fluorescein (fluorescein-5-isothiocyanate), rhodamine (tetramethylrhodamine-5-isothiocyanate), Texas Red (TR sulfonyl chloride), BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, succinimidyl ester), NBD (succinimidyl 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate), and cyanine dyes Cy3, 5, 7 (sulfoindocyanine succinimidyl ester). Arrows indicate the reaction sites.



Trade mark	Structure	λ_{abs}	λ_{em}
BOBO 1 (3)	α $X_{1(3)}$ $(CH_2)_3$ $X_{1(3)}$ α $4I^-$	462 (570)	481 (602)
BOPRO 1 (3)	α $X_{1(3)}$ CH_3 $2I^-$	462 (575)	481 (599)
JOJO 1	δ Y_1 $(CH_2)_3$ Y_1 δ $4I^-$	529	545
JOPRO 1	δ Y_1 CH_3 $2I^-$	530	546
LOLO 1	ε Y_1 $(CH_2)_3$ Y_1 ε $4I^-$	565	579
LOPRO 1	ε Y_1 CH_3 $2I^-$	567	580
POPO 1 (3)	β $X_{1(3)}$ $(CH_2)_3$ $X_{1(3)}$ β $4I^-$	434 (534)	456 (570)
POPRO 1 (3)	β $X_{1(3)}$ CH_3 $2I^-$	435 (539)	455 (567)
TOTO 1 (3)	α $Y_{1(3)}$ $(CH_2)_3$ $Y_{1(3)}$ α $4I^-$	514 (642)	533 (660)
TOPRO 1 (3,5)	α $Y_{1(3,5)}$ CH_3 $2I^-$	515 (642,748)	531 (661,768)
YOYO 1 (3)	β $Y_{1(3)}$ $(CH_2)_3$ $Y_{1(3)}$ β $4I^-$	491 (612)	509 (631)
YOPRO 1 (3)	β $Y_{1(3)}$ CH_3 $2I^-$	491 (612)	509 (631)

Figure 7. Published structures of some intercalating fluorescent dyes. Trademarks and schemes of basic structures of cyanine dyes together with their absorption λ_{abs} and emission λ_{em} maxima are summarized in the table.

yellow.[195] Use of new cyanine dyes, thiazole orange dimer (TOTO),[195,371,386,387] oxazole yellow dimer (YOYO),[195,371,386,387] and other commercially available dyes such as TOTO-1,[388,389] YOYO-1,[372,388,390] POPO-3,[372] YOYO-3,[372] YO-PRO-1,[215,391,392] TO-PRO,[388,393,394] and SYBR green 1[274,393,395−398] has been reported. An overview of the published structures of selected intercalating dyes is presented in Figure 7.

Several advantages of near-infrared fluorescence detection have been reported.[345,399−402] The motivation for using the photoprocesses of absorption and emission occurring above 700 nm includes significant reduction in scattering effects and a smaller background due to impurities. Moreover, the instrumentation is simpler and cheaper due to the advent of semiconductor diode lasers and availability of avalanche photodiodes used in optoelectronics. The impressive detection sensitivity of such simple instrumentation was demonstrated by single-molecule detection in the near-IR region[345,403] using the IR140 (5,5′-dichlor-l,l-(diphenylamino)-3,3′-diethyl-l0,12-ethylenethiatricarbocyanine perchlorate) dye (Exciton Chemical Co., Inc., Dayton, OH).

Another approach for increasing the accuracy and resolution in DNA sequencing is represented by time-resolved fluorescence. This method relies on the discrimination of the labels on the basis of their fluorescence lifetimes.[402,404−409] The commonly used dyes for DNA sequencing, which are covalently attached to a primer, exhibit fluorescence decay times from 1 to 5 ns.[407] The semiconductor lasers used can be operated in a pulsed mode and exhibit several advantageous features.[405,406] The pulsing can easily be performed at a repetition rate from 20 to 100 MHz with light pulses of less than 500 ps. By using time-resolved fluorescence with specially synthesized rhodamine derivatives the maximum sequence read length of 660 bp has been reported.[410] The detection system used employs semiconductor technology only.

An essential improvement in LIF detection was realized by the implementation of resonance fluorescence energy transfers (ET), which proved to be superior in DNA sequencing and other diagnostic techniques.[411] ETs utilize complex fluorophores consisting of both a donor and an acceptor component. The laser light excites the donor, which directly transfers its energy to the acceptor via a nonradiative pathway, i.e., without emission of a photon. This transfer occurs through interactions between the molecular orbitals of the participating molecules. Thus, ETs can also be used to measure distances within or between molecules over a range of 1−10 nm. Applications in the structural analysis of biopolymers, cell surface mapping, detection of mutations, genetic typing, and various hybridization techniques includ-

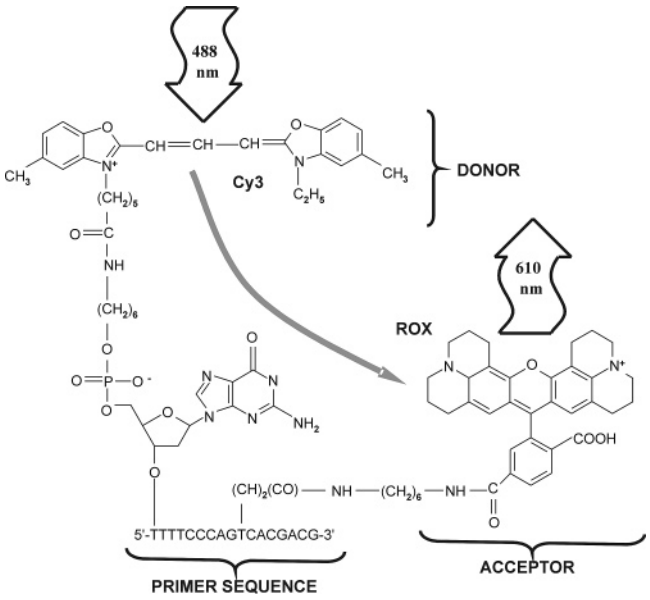


Figure 8. Chemical structure of sequencing primer labeled with resonance fluorescence energy transfer. Donor (Cy3) and acceptor (ROX) dyes provide a high absorbance when excited at 488 nm and distinct fluorescence emission at 610 nm, respectively. The arrow indicates nonradiative transfer of energy. Ten nucleotides between donor and acceptor in primer sequence are optimum for high fluorescence intensity.

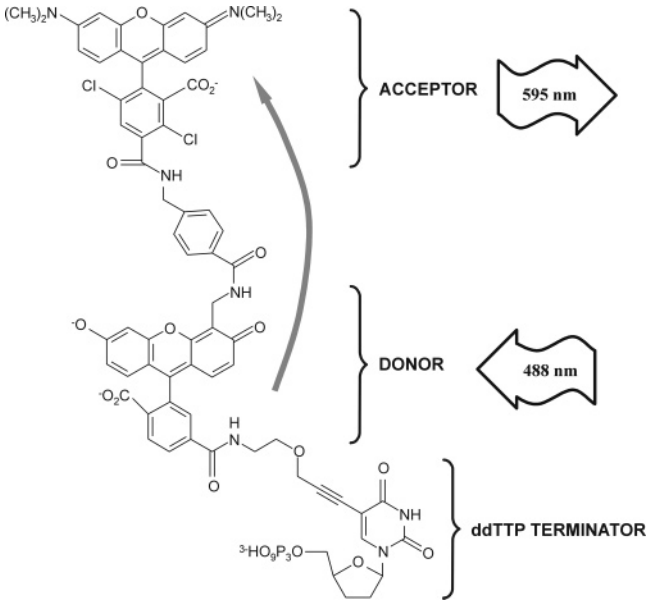


Figure 9. Chemical structure of dideoxy terminator labeled with resonance fluorescence energy transfer. Donor (6-carboxy-4′-aminomethylfluorescein) and acceptor (5-carboxy-4,7-dichlororhodamine) dyes provide a high absorbance when excited at 488 nm and distinct fluorescence emission at 595 nm, respectively.

ing the diagnostic application of molecular beacons are described elsewhere.[412,413] ET fluorescent dyes have been developed for DNA sequencing to overcome the problems with low molar absorbances of some fluorophores at a single common excitation wavelength.[414] An example of a primer fluorescently labeled by ET is shown in Figure 8. Here, the donor (Cy3) and acceptor (ROX) dyes result in a high absorbance when excited at 488 nm and a distinct fluorescence emission at 610 nm, respectively.[415]

Surprisingly, the Cy3 is a more effective donor than FAM, although its fluorescence emission quantum yield is 10 times lower. There are two reasons for this finding. First, the molar absorbance of Cy3 at 488 nm is 2.4 times higher than that of FAM. Second, the rate of radiationless energy transfer to the acceptor fluorophore is much faster than the rates of competing de-excitation pathways for the donor singlet excited state. Thus, dyes with low fluorescence quantum yields but high absorbance coefficients can be used as highly efficient donors in ET primers. The fluorescence intensity of this ET primer is 24 times stronger than that of the corresponding primers labeled with only a single acceptor dye.[415,416] Since, as stated above, the rate of transfer depends on the distance between the donor and acceptor a series of primers with systematically varied spacing was investigated.[417,418] The strongest fluorescence was observed when the number of nucleotides between the donor and acceptor was 10.[418,419] The electrophoretic mobilities of the ET primers were found to be greater than those of the corresponding primers labeled with only one dye. The increased fluorescence intensity of the ET primers and comparable mobilities of the DNA fragments generated with the four ET primers allow four-color DNA sequencing using a single laser line at 488 nm for excitation and without the need to apply mobility shift adjustments.[414,417,418,420,421] This facile procedure for tagging primers of any sequence using ET dyes is useful for diagnostic applications such as STR and SNP fragment sizing as well as multiplex sequencing, where a variety of labeled primer sequences are required.[421,422] In addition to ET primers, ET dideoxy terminators have also been developed. The main drawback in using dye-labeled terminators is that the amounts of cycle sequencing reaction products are less even than when dye-labeled primers are used. The presence of either very small or very large peaks can result in errors in automated base calling. Rosenblum et al. successfully utilized a new linker between the dye and the nucleotide to get more even peak heights in terminator sequencing. They used ETs consisting of the 5-carboxy-D-rhodamine dyes as acceptors and the 5- or 6-carboxy isomers of 4′-aminomethylfluorescein as donors.[423] The structure of the ET terminator with the optimum excitation and maximum emission wavelength is presented in Figure 9.

These dyes are the components of BigDye terminators (PE Applied Biosystems, Foster City, CA), which are widely used for sequencing now. The Amersham group (Amersham Pharmacia Biotech, Piscataway, NJ) also published the design, synthesis, and evaluation of a four-color set of energy-transfer dye terminators for high-throughput DNA sequencing.[424−426]

2.3.2. Detection Systems

Most of research activity devoted in recent years to CE detection systems has focused on LIF detectors.[427−429] However, development of electrochemical[430−434] and UV absorbance[435−437] detection systems suitable even for DNA sequencing has also been reported. Use of MS detectors connected to CE systems has become increasingly popular in recent years.[40,438] In the following paragraphs, because of its great importance in medical diagnostics, we will concentrate primarily on the current progress in LIF detection of labeled DNA.[439] Detection systems for combining CE with LIF may be divided into several groups according to the principle used. The simplest arrangement consists of single laser on-column excitation with collection of the fluorescence emission at right angles using a microscope objective. Figure 10, shows an arrangement with two objectives and two beam

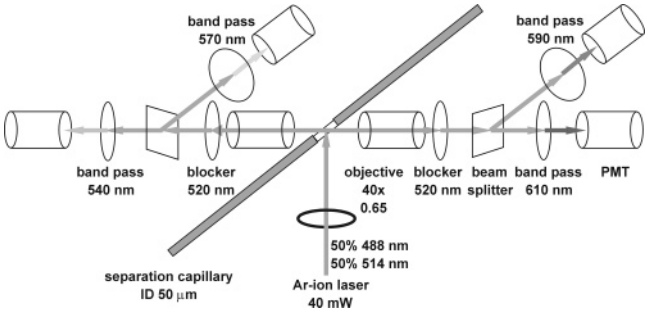


Figure 10. Scheme of a four-channel LIF detector. Fluorescence emission is collected by two microscope objectives placed at a right angle to the separation capillary and excitation laser beam. Filters behind objectives block scattered laser light and transmit only selected wavelength.

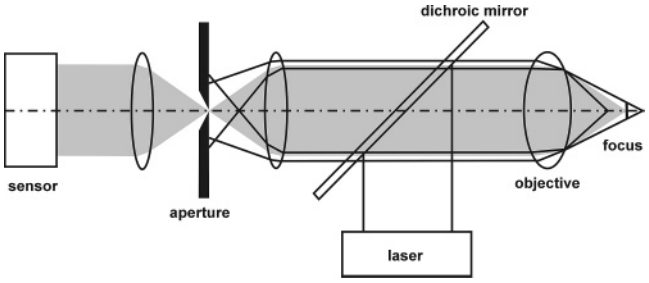


Figure 11. Confocal detector scheme. Laser excitation light is reflected by a dichroic mirror and focused by an objective on the specimen. Fluorescence emission is collected by the same objective, passes the dichroic mirror, is spatially filtered by pinhole optics, and is focused to the sensor.

splitters, which is preferable for high collection efficiency. The emission is spatially and spectrally filtered once it has passed through the microscope objective in order to remove scattered excitation light. Subsequently, it is split into four beams and filtered again to select a wavelength region of interest. The fluorescence is detected with a photomultiplier tube (PMT) or a semiconductor detector. Many CE sequencing detection systems use either a single laser operated in multiline mode[307,440−442] or two excitation wavelengths from two lasers[309,443,444] in order to increase the detection sensitivity of fluorophores differing in molar absorbance maxima. Such an arrangement was used for the peak identification and base calling in the CE-LIF system with two-color excitation using an Ar-ion laser (488 and 514 nm) and a single-element detector.[442]

More sophisticated detection systems can include a confocal detector. Here an epi-illumination approach where the laser beam is focused on the capillary by a microscope objective and the fluorescence emission is collected by the same objective followed by confocal detection is used.[294,445−450] The key element of such an approach is use of spatial filtering, or pinhole optics, which eliminates out-of-focus light (see Figure 11). Using this technique the laser scattered light is also eliminated, which is important for reaching a high-sensitivity LIF detection.

Confocal detectors are widely used for single-molecule detection.[346,350,403,405,406,451] The spatial arrangement of the confocal detector is ideal for scanning the signal from parallel lines on slabs,[382] microfluidic devices,[194,224,450,452,453] or bundles of multicapillary systems.[197,447,454,455] Either the objective or a separation device is placed on a moving stage and the signal is registered at individual positions. The translation stage moves at 1 cm/s in a direction perpendicular to the migration path, and the separation is registered at a

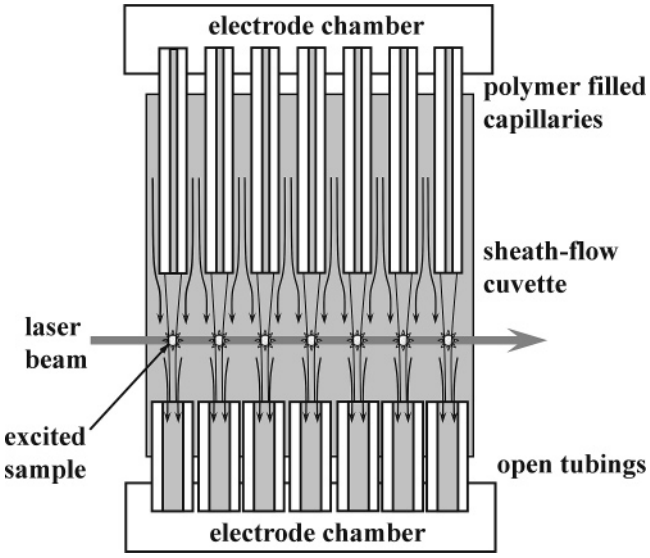


Figure 12. Scheme of sheath-flow cuvette. Analytes leaving separation capillaries, confined into wall-less cells, are transported downstream by flow. Laser beam is focused on sheath flow chamber beneath capillary tips. The lower tubes provide electric contact with the anode electrode chamber.

frequency of 2 Hz.[454] When a four-color fluorescence signal is detected it is first divided into four beams by dichroic beam splitters and then focused through a pinhole to PMTs. A confocal detection system is used in the commercially available microfluidic Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).[456]

One way to eliminate the laser scattering from capillary walls is to position the detection spot behind the capillary outlet. In this sheath-flow detector, originally developed for use in flow cytometry,[457] the analyte is transported by the flow along the capillary downstream of its outlet and detected beneath the capillary tip confined in a wall-less cell.[344,458−461] The scheme in Figure 12 demonstrates the compatibility of this arrangement with capillary arrays. The laser beam is focused on the sheath flow chamber, a 1 mm wide gap between the arrays of the separation capillaries, and 0.2 mm i.d. tubes. The lower tubes provide not only a proper hydrodynamic flow of a buffer but also an electric contact with the anode electrode chamber. The fluorescence emission is collected at right angles by an appropriate system of lenses and is registered by a two-dimensional detector, a CCD camera.[363,460,462−468] Thus, the technique enables both high sensitivity and high-throughput analyses and has been incorporated into the development of the ABI Prism set of capillary sequencers (ABI Foster City, CA).

3. CE Methodology for DNA Diagnostics

Although research activity in the field of direct detection of DNA sequence variants on a single-molecule level increases every year,[48,54,351,469−476] the methodologies currently implemented in laboratory practice are based on two-step procedures processing typically hundreds of nanograms of a template DNA. In the first step a DNA template is treated by a molecular biology procedure to produce samples with characteristic sizes or conformations. Frequently, part of this treatment is the incorporation of fluorescent or binding tags. Individual segments of investigated sequences are amplified and/or distinguished by the DNA modifying enzymes, such as polymerases, restrictases, and ligases, or

by hybridization with specific oligonucleotide probes. In the second step the samples are analyzed using a spectroscopic or separation method. Thus, DNA fragments of a specific size or conformation labeled with a specific tag can easily be separated, detected, or even identified. In principle, the methods can be divided into two categories: hybridization (array-based) and separation (gel-based) methods. Many DNA diagnostic methodologies have already been adapted for CE techniques. Examples of such CE applications well described in the current literature include mutation detection or gene-polymorphism analysis based on restriction fragment length polymorphism (RFLP) or length polymorphism of PCR-amplified fragments (AFLP), single-strand conformation polymorphism (SSCP), heteroduplex analysis, constant denaturant (CDCE) or denaturing gradient capillary electrophoresis (DGCE), single-nucleotide primer extension (SNuPE), and DNA−DNA hybridization. Most effort, however, has been put into DNA sequencing. Development of these diagnostic applications has been reviewed in several publications.[18,23,59,477−482]

3.1. Sample Preparation

Molecular diagnostics has become a rapidly evolving area of clinical testing over the past two decades. In particular, recombinant DNA technologies such as polymerase chain reaction (PCR) amplification and the Sanger−Coulson DNA sequencing method, DNA hybridization, restriction cleavage by endonucleases, and cloning have opened up new possibilities for development of DNA diagnostics with high sensitivity and selectivity. Together with the miniaturization of analytical systems, sample preparation techniques have also been adapted for ultrasmall quantities and integrated with micromachined devices.[26,77,82,84,483−487]

3.1.1. Restrictase Cleavage

One of the major breakthroughs in molecular biology was the discovery and use of DNA restriction enzymes. Restriction DNA endonucleases are bacterial enzymes ranging in size from 157 (*Pvu* II) to 1250 (*Cje* I) amino acids that attach to DNA and cut both strands at sites of specific base sequences.[488] For example, restriction enzymes *Eco*RV from *Escherichia coli* and *Hind* III from *Haemophilus influenzae* recognize the sites GAT↓ATC and A↓AGCTT, respectively, and cleave the DNA chain at the positions indicated by the arrows. Hundreds of restriction enzymes are available for use in various combinations for identifying portions of a gene by cutting it at specific base sequences into discrete sized fragments. In the majority of cases, size-based separations of the fragments obtained through cleavage with two restrictases, both individually and together, provide sufficient information to deduce the position of the restriction sites and construct a characteristic physical map of the chromosome or gene. The presence of deletions or insertions in the genome results in the polymorphism of restriction fragments lengths (RFLP). Such polymorphism can be detected as a shift, appearance, or disappearance of peaks in an electropherogram of digested DNA.

3.1.2. PCR Amplification

PCR amplification is a technique of key importance in DNA diagnostics. Nowadays, nearly all mutation detection methods rely on PCR amplification. This technology allows the detection and multiplication of a selected region of genomic or cDNA, which can be then analyzed. The principle behind PCR involves a series of amplification reactions performed in cycles over a short segment of the DNA (see the scheme in Figure 13). The basic reaction has three phases—denaturing, annealing, and extension. The main components of the reaction mixture are the double-stranded DNA (dsDNA) of interest, specific oligonucleotide primers, and thermostable DNA polymerase (Taq polymerase). The process begins with heating of the mixture, which causes dissociation of the two DNA strands. In the second stage the reaction mixture is cooled to allow the primers to anneal to their complementary sites on the single-stranded DNA (ssDNA) molecules. Thus, in an ideal case the entire DNA in the reaction mixture should be single-stranded except the regions where the primers are base paired. The third stage of the process is primer extension, in which the mixture is heated again and the thermostable DNA polymerase directs the synthesis of a new DNA strand in the 5′ to 3′ direction as an elongation of the primer sequence. At the end of each PCR cycle the newly synthesized DNA extension product will serve as a DNA template for subsequent cycles. Thus, the DNA copies accumulate exponentially and the amplified segment of DNA can be isolated from the other fragments using gel electrophoresis.

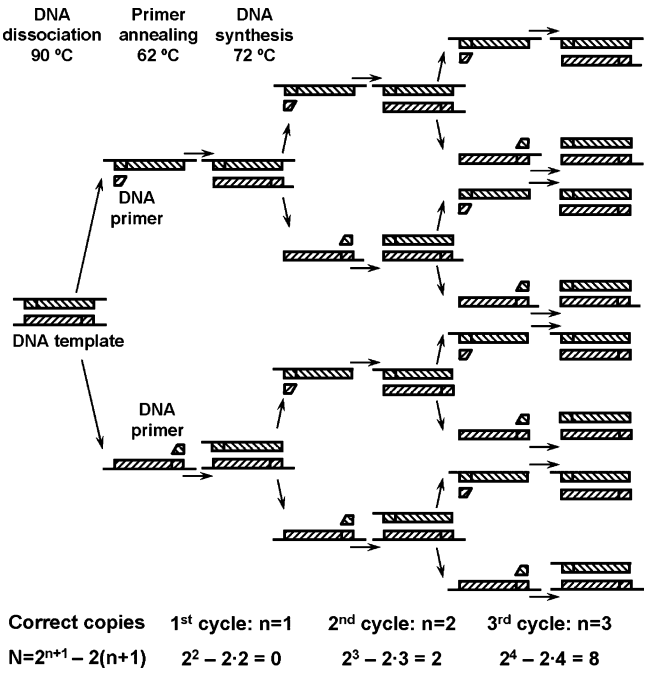


Figure 13. PCR amplification scheme. Series of amplification reactions are performed in 20−40 cycles over a short segment of template DNA. Three phases of reaction—denaturing, annealing, and extension—are controlled by temperature cycling. The polymerase reactions start at specific oligonucleotide primers annealed to their complementary sites on ssDNA molecules. Newly synthesized extension products serve as templates for subsequent reactions. Thus, the DNA copies accumulate exponentially.

The length of PCR fragments is determined by the size variations between sites complementary to PCR primers or generation or elimination of such sites. Multiplex PCR makes it possible to amplify several separate sequences simultaneously in a reaction mixture. There are potentially two different approaches for the miniaturization of the PCR in microfluidic devices: the chamber type, where the rapid thermal cycling of a reaction volume of less than 1 μL is controlled,[201,398,489−497] and the flow-type, where the reaction mixture flows through a channel with segments of different

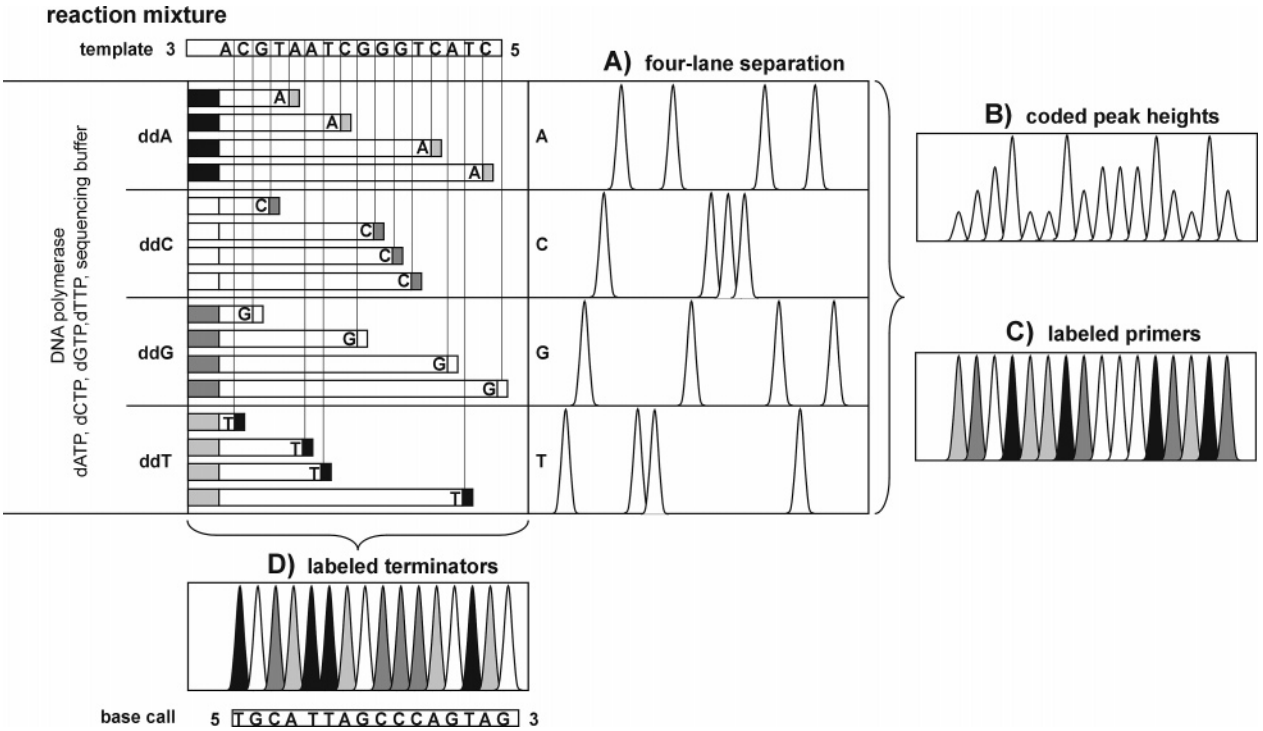


Figure 14. Sanger sequencing reaction scheme. Four reactions are performed separately with different dideoxy terminators ddA, ddC, ddG, and ddT and the same 5′ dye-labeled primer. Separation strategies: (A) reaction products are separated in four lanes, and the migration times are compared; (B) products of the separate reactions can be pooled and analyzed in a single lane, provided the ratios between deoxy and dideoxy nucleotides in the individual reactions differ, and consequently, peak heights serve as markers of individual bases; (C) similarly, four differently labeled primers in individual reactions code the bases; (D) if fluorescently labeled dideoxy chain terminators are used, only one sequencing reaction is needed, since the specific label is associated with the base.

temperature.[498,499] PCR amplification integrated onto diagnostic or analytical chips can also be performed.[69,82,86,485,486,500]

3.1.3. Sanger Sequencing Reaction

The chain termination dideoxy method, now used nearly exclusively for the preparation of DNA sequencing fragments, was developed by Sanger's group in 1977.[501] In 1986, the original radioactive labeling was replaced by a fluorescent one, which can easily be detected and automatically analyzed at the time of separation.[440,502,503] Sanger's reaction scheme together with various labeling strategies are presented in Figure 14. In principle, this reaction transforms the order of a base in a DNA template sequence into the length of a respective fragment terminated by a complementary base. Thus, the sequence can be determined by a size-specific separation of the sequencing fragments. Similar to PCR, the sequencing reaction is based on the enzymatic synthesis of a complementary chain on a template DNA. The difference is that only a 3′ primer is used and the synthesis of a single strand is randomly terminated by the dideoxy nucleotides. First, the sample is heated to 95 °C to dissociate the two strands. Then, the temperature is reduced to 50−60 °C so that the primer can anneal to one strand, namely, the template. At 70 °C a thermostable polymerase forms a complex between the template and the 3′ end of the primer and extends it by sequentially incorporating deoxynucleotides one base at a time. The incorporation rates are a few hundred bases per second. The labeled strand is extended until a dideoxynucleotide is randomly incorporated and the extension is terminated, since the OH group at 3′ is missing in the dideoxy form. In cycle sequencing, the reaction is performed in 20−30 cycles on the same template. Thus, the quantity of sequencing fragments increases linearly unlike PCR where it increases exponentially.

3.2. DNA Sequencing

Determination of the genome sequence of an organism provides complete information toward the understanding of its genetic basis. Thus, development of effective sequencers represented the most attractive challenge for analytical chemists in the past decade.[20,504−506] DNA sequencers based on capillary array electrophoresis (CAE), namely, the ABI PRISM models (ABI Foster City, CA)[507−509] and the MegaBACE models (formerly Molecular Dynamics Inc., Sunnyvale, CA),[510,511] are the best-selling products in the history of analytical instrumentation. Clearly, progress in the automation of DNA sequencing was of paramount importance for HGP. As early as 1986, Smith and co-workers in Hood's laboratory developed a method for the partial automation of DNA sequence analysis with real-time detection.[440] They used LIF detection of fluorescently labeled sequencing fragments migrating in glass or quartz tubing of 1−2 mm i.d. filled with an 8% polyacrylamide gel. Some of this technology was incorporated into partially automated sequencers employing electrophoresis in ultrathin slabs (ABI 373, 377 sequencers, Applied Biosystems, Foster City, CA).[305] Before the decision to implement CE sequencers in the HGP, several major improvements in CE instrumentation for DNA sequencing were accomplished. The cooperative development of a variety of new analytical instruments, methods, and reagents for nucleic acid analysis increased its productivity and reliability. Compared with classical and ultrathin SGE, narrow-bore capillaries allowed for higher speed and resolution of separations. Moreover, the capillaries were suitable for the full automation of sample injection and

loading of separation medium. The following items were all essential for the development of large-scale sequencing: (i) systems for data acquisition and evaluation (base calling) at the time of analysis, (ii) detection systems compatible with CAE, (iii) fluorescent labels with suitable chemistry, (iv) replaceable sieving matrices, and (v) suitable denaturing and separation conditions.

The simplest way to determine a sequence is to perform four reactions separately with different dideoxy terminators and the same 5′ dye-labeled primer. The reaction products are then separated in four lanes, and the migration times are compared as shown in panel A in Figure 14.[502] The products of the separate reactions can also be pooled and analyzed in a single lane, provided the ratios between deoxy and dideoxy nucleotides in the individual reactions differ (panel B). In such cases, the quantities of the individual products will also differ, and consequently, the peak heights can serve as markers for the individual bases.[309,399,447,512−517] However, using the spectra of four-color sequencing reactions rather than the peak heights provides more information for peak and base identification using a base-calling software. These reactions can be performed either with primers[440] or dideoxy chain terminators[503] labeled with four different fluorescent dyes. The reactions with labeled primers must be done separately and can be pooled before separation (panel C). If fluorescently labeled dideoxy chain terminators are used, only one sequencing reaction is needed, since the specific label is associated with the base (panel D). Another advantage of the use of the labeled terminators is that only properly terminated fragments are labeled. Thus, strands resulting from sudden stops associated with polymerase pausing or those falling off the template without proper termination with the labeled terminator are not detected. In summary, DNA sequencing using four-color labeled dideoxy terminators provides clear advantages, and most sequencing today is done by this method.

An important issue of a large-scale DNA sequencing is the selection of a convenient sequencing strategy for assembling many relatively short reads, the results of CE analysis. The most common approach used for sequencing of fragments of sizes of several kilobases is the primer walking strategy. Here, sequencing primers are systematically designed to anneal to the known sequence unidirectionally step by step as the new segments of the template are consecutively sequenced. However, this laborious and time-consuming method cannot be implemented in the HGP. The whole-genome shotgun sequencing strategy proved to be much more convenient.[518] In simple terms, chromosomal DNA is sheared randomly (physically or enzymatically) into pieces which are cloned into plasmids and sequenced on both strands. The sequences of the individual fragments that are obtained are then analyzed for overlaps, aligned, and assembled into a final sequence by computer software. Accidental gaps must be sequenced systematically. The application of the whole-genome shotgun sequencing method is considered as one of the crucial events in accelerating the HGP.[519]

DNA sequencing is the most successful application of CE both scientifically and commercially.[27] The HGP with its goal of sequencing the entire human genome began in 1990 and was scheduled to last 15 years. However, it was completed under budget and in only 10 years mainly due to the use of automated CAE sequencers. It is pertinent to present some facts just to demonstrate the true powers of such advanced automated CE instrumentation. Celera Genomics, the company where the advanced technology for the human genome sequencing was developed, used 300 of these 96-capillary ABI PRISM sequencers (model 3700). They generated a 14.8 billion bp DNA sequence from 27 271 853 high-quality sequence reads (550 nt) in 9 months in the year 2000.[519] Thus, the 5.11-fold coverage of the genomes of five individuals was attained. This project generated a 2.91-billion bp consensus sequence of the euchromatic portion (rich in gene concentration) of the human genome and is considered to be both the largest in scope and the most challenging in biology to date.

Development of CE instrumentation and methodology for DNA sequencing resulted in the design and construction of high-performance multicapillary commercial devices ultimately successfully implemented not only in the HGP[519,520] but also in a range of other applications such as the whole genome sequencing of drosophila,[521] mouse,[522] chimpanzee,[523] and others.[524,525] The cost of these applications, however, is still too high for routine sequencing tasks. Therefore, microfabricated devices enabling many parallel and fast separations were expected to bring a further improvement in the throughput and costs of analyses based on the Sanger sequencing chemistry.[74,76,78,82,83,85,239,450,487,526,527] Nevertheless, the increasing demands for genomic sequencing data stimulated the investigation of completely new sequencing strategies that promise to bring an exceptional increase in productivity.[25,28,47,53−55,351,474,528,529] The most successful methods exploit the monitoring of sequencing by using synthesis in highly parallel systems of thousands of samples.[47,54] Such methods can achieve an approximately 100-fold increase in throughput over the current Sanger sequencing technology. Thus, the idea of sequencing a human genome sequence for 1000 U.S. dollars could become reality.

3.3. DNA Fragments Length Polymorphism

The most frequent application of CE in DNA diagnostics is the analysis of the length polymorphism of DNA fragments derived from genomic DNA. There are two principal methods for generating such fragments: digestion of genomic DNA by specific restriction endonucleases and PCR amplification. In the first method, known as restriction fragment length polymorphism (RFLP), the choice of individual enzyme or a combination of two or more determines the size of the genome fragments. The second method, amplified fragment length polymorphism (AFLP), uses PCR amplification to synthesize the fragments on genomic DNA with sizes determined by the choice of the primers (see Figure 15a). In both methods, the length polymorphism can be detected as a mobility shift of zones of fragments with different sizes resulting from mutations caused by deletions (Figure 15b) or insertions (Figure 15c) in a genome. A combination of both methods is used too. In that case, a long fragment spanning a region with multiple mutations is amplified first and then digested by a restrictase.[211] The methods are especially sensitive when polymorphisms or mutations affecting a restriction site (RFLP) or a site complementary to the used PCR primers (AFLP) are detected. Such polymorphisms can result in either the loss (Figure 15d) or the gain (Figure 15e) of a restriction or primer site leading to striking differences in fragment length; compare peaks a, d, and e in Figure 15.

The RFLP method is used not only for mutation detection but also as a basis for the construction of restriction (or

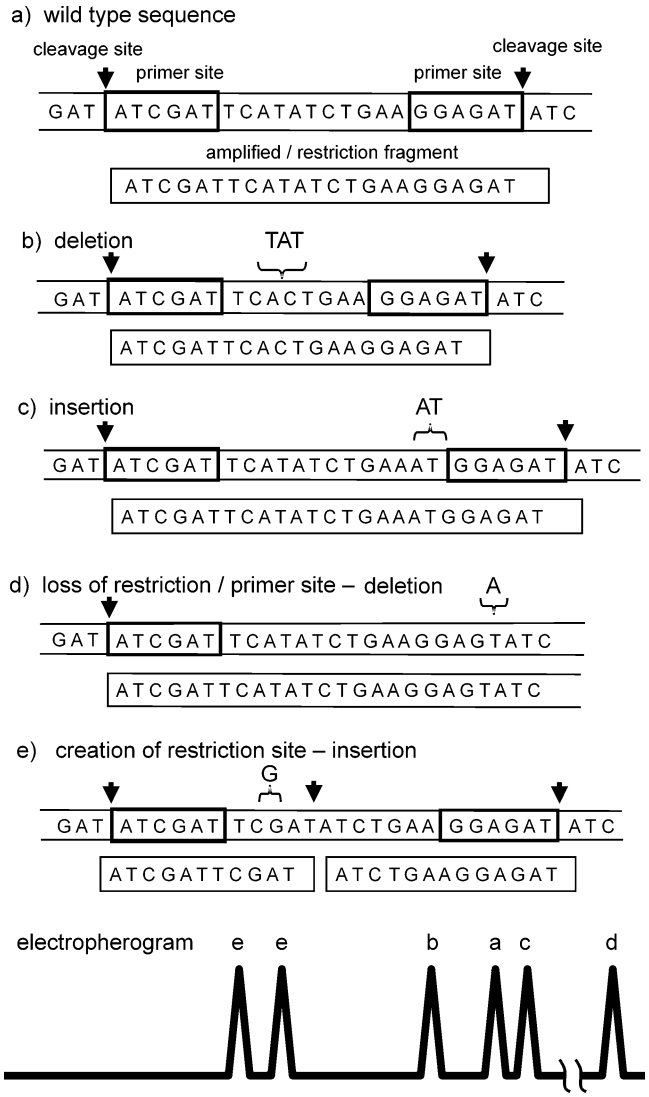


Figure 15. Scheme of RFLP/AFLP technique. Size-based separation reveals (a) wild-type sequence, (b) deletion, (c) insertion, (d) loss of restriction (GAT↓ATC) and primer (GGAGAT) site, and (e) creation of restriction site (GAT↓ATC) in the investigated segment of DNA.

physical) maps of various genomes. Restriction maps provide concise characteristics of a given genome rather than its complete sequences; hence, they are particularly useful for the molecular identification of microorganisms.[530−534]

The main advantage of CE for molecular size analysis is its high reproducibility, precision, and accuracy. Due to the high separation efficiency, peaks representing individual DNA fragments are very sharp. Hence, even two fragments differing only by a single nucleotide can be well resolved and their migration times precisely determined in fully automatic systems with data evaluation.[373,535−538] High separation efficiency also facilitates the separation of samples prepared by multiplex PCR amplification from several alleles in a single electrophoretic run.[203,539−542] As in chromatography, various types of programming, such as electric field strength, current, power, or temperature, can easily be employed to increase the resolving power of CE separations.[289,543−547]

Separation in sieving media does not provide absolute values of DNA mass or base pair count. A calibration method is therefore needed to transform the migration time values or effective electrophoretic mobility into molecular size. In some instances an on-line combination of CE with MS can help this precise determination.[40,438] The most frequently used option is to use calibration by the addition of size standards.[98] In this case, however, the sequence-dependent migration of dsDNA fragments must be taken into account.[548−551] The GC-rich fragments migrate anomalously due to significant changes in their conformations.[551−553] Even the free solution mobility of these DNA fragments can be affected by the changes.[554] This phenomenon is more pronounced in LPA than in agarose solutions, and it is strongly dependent on the concentrations of sieving media and temperature.[289,555,556] Intercalating dyes can also induce significant shifts in mobilities.

Another application of size-based separations in DNA diagnostics is the analysis of tandem repeat polymorphism. This type of polymorphism is characterized by repetitive DNA sequences arrayed as tandem repeat units, mostly in the noncoding regions of a genome. Two classes of such sequences, minisatellites (VNTR) and microsatellites (STR), have been distinguished and described in the literature. These polymorphic regions are typically flanked by non-polymorphic DNA sequence. Thus, PCR amplification with primers bordering the polymorphic repeat region will produce DNA fragments of varying lengths determined by the number of repeats. To avoid an error in DNA size determination due to the sequence-dependent migration, allelic ladders are used as the absolute standards.[260,269,333,538,557,558] A relative mobility shift evaluated with the help of internal allelic standards or heterozygotic samples yields more reliable results for AFLP and STR polymorphism analyses. Nevertheless, fluorescent labels of allelic ladders can also be the cause of errors in the determination of the numbers of repeated units.[559]

Several papers have demonstrated better separation selectivity of ssDNA fragments when compared to their ds forms.[105,560] Therefore, fast and high-resolution separations can be accomplished under denaturing conditions. Fluorescently labeled tetranucleotide STR standards were successfully analyzed under denaturing conditions at 60 °C within 30 min. The average resolution obtained was 1.4 bases for a 200 bp fragment with a standard deviation of 0.2 bases.[203] As a result of optimization, polymorphic fragments carrying STR regions of sizes 299 and 300 nucleotides could be distinguished. However, the separation speed tends to be lower due to the increased viscosity of the denaturing agent. This analysis was accomplished in a 2% solution of HEC in 1×TBE buffer with 6 M urea and 10% formamide in 50 min. Moreover, no extra peaks due to formation of heteroduplexes were observed.[203] To overcome problems with incomplete denaturing and the viscosity of urea, a highly alkaline separation medium was used for the STR analysis.[105]

3.4. Single-Strand Conformation Polymorphism

Single-strand conformation polymorphism (SSCP) is the most popular technique for the diagnostic screening of mutations and polymorphisms in small segments of DNA.[59,479,480,561−566] Mutations are detected by monitoring mobility shifts of individual DNA strands caused by their conformational changes. The main advantage of SSCP is its capability in the sensitive detection of mutations (substitutions, small deletions, and insertions) at various positions in DNA fragments. Even substitutions of a single nucleotide give rise to conformational changes under nondenaturing conditions and result in a mobility shift. The scheme in Figure 16 outlines the principle of the SSCP technique. The

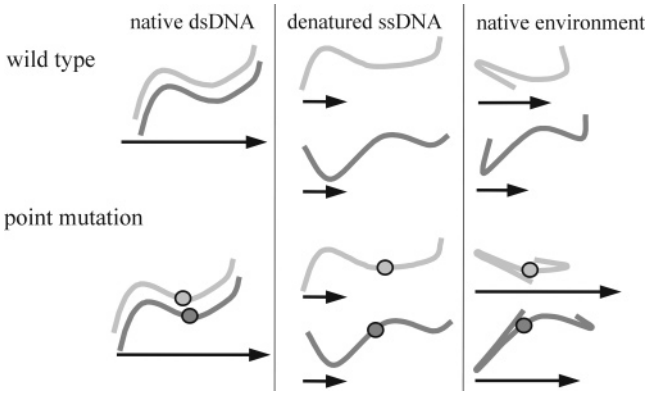


Figure 16. Scheme of SSCP technique. Conformation and, consequently, electrophoretic mobility of ssDNA fragments in the native environment are sensitive even to point mutations. Arrows characterize respective magnitudes of effective electrophoretic mobilities.

arrows under the conformation structures represent their effective electrophoretic mobilities. The scheme indicates that a point mutation does not affect the electrophoretic mobilities of dsDNA fragments under native conditions as well as the mobilities of their individual strands under denaturing conditions. The mobilities of dsDNA fragments are higher than those of their ss analogues mainly due to their double charge. When introduced into nondenaturing conditions, the ssDNA adopts a folded structure stabilized by an intramolecular duplex between complementary regions and/or base stacking within each strand. Consequently, the folding and structural conformation are altered by the presence of mutations as well as the nucleotide sequence in the vicinity.

Since the mobility is determined by the conformation of the DNA fragment as a whole, the sensitivity of mutation detection decreases with its length. It is evident that in long fragments the structural changes induced by the mutation are masked while the probability of the presence of a polymorphic background increases.[567] Thus, the optimum lengths of fragments analyzed by SSCP span the range from 100 to about 400 nt, although successful detection of point mutations in fragments as long as 741[568] and even 1223 nt[569] have been published.

In practice, the sample is denatured prior to analysis and the ssDNA fragments are introduced into the nondenaturing sieving medium and separated by electrophoresis. The denaturing can be accomplished chemically under highly alkaline conditions[570−572] or by formamide[573] physically by heating or by a combination of both methods.[574] Nowadays, the preferred method is by heating the sample to a temperature over 90 °C for 1−5 min followed by immediate chilling on ice. The SSCP methodology was originally used in conjunction with polyacrylamide SGE.[570,573] However, CE in replaceable sieving media has been shown to have several advantages over SGE even for SSCP.[575−580] These advantages include short analysis time, full automation of parallel analyses in capillary arrays, small sample volume, minimal reagent consumption, highly sensitive LIF detection, and, most importantly, high separation efficiency. High separation efficiency is the prerequisite for detecting even very subtle shifts in the mobility of a mutant fragment.[566,581−585] Since SSCP relies on the changes in DNA conformation, it is very sensitive to the physicochemical properties of the separation medium. The most frequently used replaceable sieving media are derivatives of LPA[586] (PDEA, PDMA[587]), cellulose (HEC[588]), and commercial polymers: Genescan[572] and POP[569,589] (Applied Biosystems, Foster City, CA). Precise temperature control of the separation capillary allows the operator to adjust the maximum selectivity and maintain good reproducibility between experiments.[568,586,590−596] Electric field strength and concentration of a separation medium, which exert a mechanical stress on the migrating structure, also affect the resolution of individual conformers.[568,571,586,589,591,597] For the separation of fragments of a relatively short size (shorter than 400 nt), a sieving polymer of molecular mass lower than 600 kDa provides good selectivity and an acceptable viscosity at concentrations up to 6%.[586,598] An increase in the separation selectivity by adjusting the pH of the background electrolyte[593,594] and/or the concentration of various additives[578,586,587,592] has also been reported. The effect of temperature and pH varies for different fragments.

Additives to sieving gels such as glycerol, glucose, or sucrose are frequently used, yet their effect on the separation selectivity remains ambiguous. These polyols probably act as weak denaturants, partially opening the folded structure of ssDNA.[574] As in the case of SGE, addition of glycerol to the replaceable sieving media in CE separations has been reported as enhancing the resolution[578,587,592,599,600] as well as decreasing it in some instances.[586] Borates present in the sieving media are known to form monomeric and dimeric complexes with polyols such as glycerol, monomeric sugars, polysaccharides, or DNA and RNA. Such transient interactions can result in an improvement of the sieving properties through stabilization of the polymer entanglements.[287]

It is well established and documented in the literature that the optimal detection parameters differ depending on a specific mutation as well as the size and sequence of the DNA fragment. This applies to both SGE separations and polymer solutions using CE. Hence, there are no general rules for the optimization strategies of the SSCP technique. On the other hand, it has been observed by many researchers that separations performed at temperatures over 40 °C[568,592,599] with electric field strengths over 800 V/cm[586] or concentrations greater than 8% of low molecular mass LPA[586,591] generally lead to a dramatic decrease in differences in mobility shifts of mutant fragments and in their wild-type counterparts.

There is a lack of complete theoretical understanding of the factors affecting the three-dimensional folding of ssDNA fragments under particular physical conditions. Therefore, mobility shifts during SSCP analysis are quantitatively unpredictable. Nevertheless, certain computer programs are available providing two-dimensional models of the secondary structures of individual DNA/RNA strands.[601,602] These models are quite helpful in the preliminary analysis of a separation problem and optimization of the analytical conditions.[571,599,600] A more precise quantitative description of the model of the hydrodynamically equivalent sphere of a ssDNA coil needs a complex three-dimensional analysis.

The typical records of homozygous and heterozygous ss fragments separated in a native environment are compared in Figure 17. When SSCP electropherograms are analyzed, they typically reveal three and five peaks for homozygous or heterozygous samples, respectively. However, these numbers can actually be higher for either case. The major peaks represent either the predominant two or four conformers of ssDNA and reannealed dsDNA, respectively.

However, other minor peaks are frequently observed. These peaks may be a result of different stable intermolecular

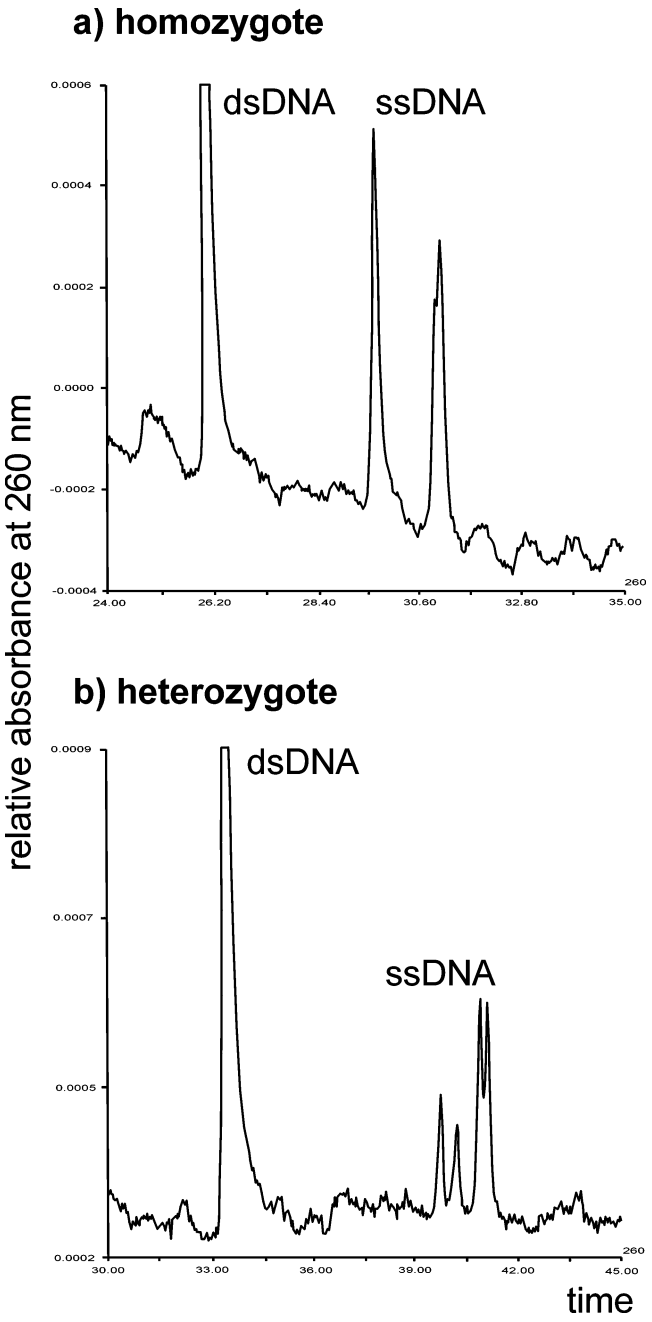


Figure 17. Typical separations of SSCP analysis. DNA fragments amplified on a genome of (a) health homozygote and (b) phenylketonuria affected individual (point mutation C > T in phenylalanine hydroxylase gene on chromosome 12). Separation conditions: 2% solution of agarose SeaPrep in 1xTBE with 10% formamide at 30 °C; L_C = 55 cm; L_D = 50 cm; E = (a) 183 and (b) 135 V/cm. Unpublished authors' result.

interactions.[603] In addition, homoduplexes, heteroduplexes, and even complexes of ssDNA and residual PCR primers could be a reason for formation of additional peaks.[604] Peaks of homo- and heteroduplexes provide additional evidence that mutations are present. Thus, a tandem SSCP and duplex analysis improves the sensitivity of mutation detection.[597,604−606] Nowadays, certain genes and point mutations in them are so well characterized that they can serve as standards in the development of instrumentation and methodology for CE-SSCP. The p53 tumor suppressor gene is one such example.[578,584,592,596,607,608]

Many methodologies have been developed for SSCP mutation detection in commercially available CE instruments with LIF detection. Detection based on fluorescence enables not only high sensitivity detection but also identification of all peaks in the electropherograms.[609] If the complementary strands of the DNA fragments are labeled using different fluorophores, electromigration of both strands can be recorded independently.[571] However, in such a case the wild-type and mutant fragments cannot be distinguished. Therefore, for the unambiguous identification of all four peaks (two alleles of two strands each) in the electropherograms of heterozygotic samples, they need to be compared with those of both healthy and affected homozygotes.[571] Absorbance detectors or a nonselective fluorescent staining are satisfactory if the goal of the analysis is only to confirm a given mutation. Nowadays, clinical laboratories routinely use commercial CE instruments compatible with the two-dye SSCP LIF technology for mutation detection.

Ultrafast SSCP analyses have been demonstrated using microfluidic devices with LIF detection. Three common mutations in BRCA1 and BRCA2 genes were analyzed in less than 120 s on a single-channel glass microchip filled with 2.5% HEC with 10% glycerol and 1 × TBE buffer. This analysis time represents a 100-fold decrease when compared to conventional SGE.[588] Two commercial devices for microchip electrophoresis, the Hitachi SV 1100 (Hitachi Electronics Engineering, Tokyo, Japan) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using chips with migration distances of 30 and 15 mm, respectively, were employed for detection of mutations in the K-ras gene. The conformers of both wild-type and mutation-carrying fragments were separated in 1.5% MC in several minutes.[587] These two examples of fast separations based on the SSCP technique proved that a reasonable resolution is attainable even with short migration distances.

3.5. Partial Denaturing Electrophoresis

Detection of low-frequency somatic mutations is of particular importance in mutation and cancer research as well as in molecular diagnostics. Denaturing gradient and constant denaturant gel electrophoreses are two DNA separation technologies which were developed to detect point mutations and rare variants of known and unknown mutations at specific DNA loci.[610,611] They are also frequently used in the search for mutations in a given locus. The capillary versions of the methods mentioned above, denaturing gradient (DGCE) and constant denaturant capillary electrophoresis (CDCE), have been successfully developed, and details about their instrumentation, methodologies, and analytical properties are presented in the literature.[612−615] In contrast to SSCP, where samples are completely denatured before analysis, in DGCE and CDCE, the native samples are injected and the separations are performed in a moderately denaturing environment. Under these conditions, DNA fragments are partially melted and, consequently, their mobilities reduced. The presence of mutations can cause a substantial change in the extent of the DNA melting and, thus, also in the electrophoretic mobility in the sieving medium. The separation selectivity of these methods is based not only on the nature and position of the mutation but also on the denaturing parameters during the migration through the separation column. As is apparent from their names, whereas in CDCE the denaturing parameters are constant, in DGCE they vary in space and/or time.[614] The advantage of DGCE is that by changing the denaturing parameters, suitable conditions for separation of the sample components are reached at a certain

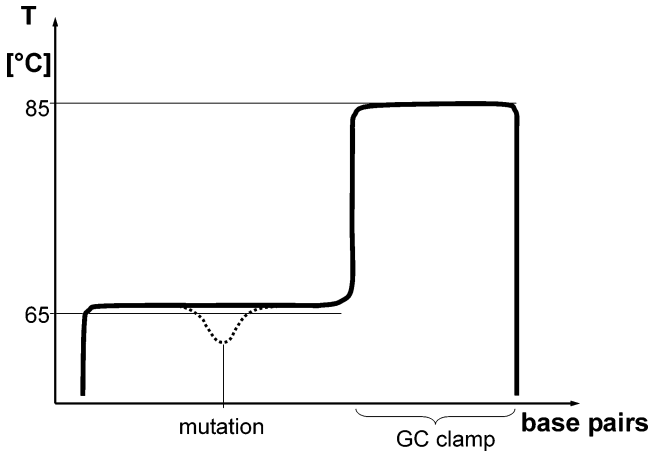


Figure 18. Schematic theoretical melting profile of a two-domain fragment with melting temperature *T* of individual base pairs plotted against their position in the fragment. Base pairing mismatch in heteroduplex causes a decrease in melting temperature as indicated by the dotted curve.

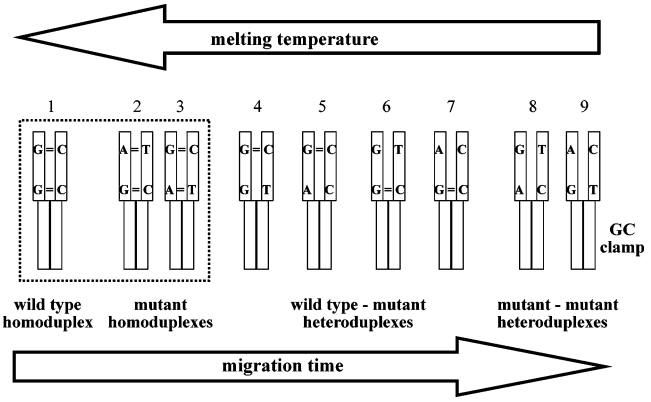


Figure 19. Scheme of nine possible duplex combinations of strands coming from one wild-type and two mutant homoduplexes. Each of the two mutant fragments carries one point mutation, the substitution of AT to GC pair, at different position. Mutation-containing heteroduplexes dissociate more easily (at lower temperature) and in a more relaxed conformation migrate through a sieving medium slower than homoduplexes.

point in the analysis. Therefore, although the parameters of the denaturing agents (i.e., temperature and concentration) do not require a precise control, the analyzed fragments are still likely to be resolved even without knowing their exact dissociation curves. One consequence is that the conditions for the separation of two particular fragments do not remain optimal throughout the whole time of the electrophoresis operation period. In contrast, CDCE attempts to set optimum separation conditions and keep them. That frequently proves time consuming and technically difficult. In practice it means that it is necessary to maintain the temperature constant within several hundredths of degrees Celsius. As reiterated above, the optimum separation conditions for a particular couple of fragments may be far less than what is optimum for others. An interesting solution to this problem is the application of pulsing temperatures designed by Minarik et al.[616,617] A rapid temperature cycling of several cycles per minute with an amplitude varying from 2 to 4 degrees provides the optimum separation conditions for each couple of fragments many times during the run. Thus, the temperature does not need to be controlled with the precision necessary in CDCE, and the resolution and reproducibility are better than that obtained in a single-sweep gradient.[618,619] Since the regular periodic temperature cycling is compatible with multiple consecutive sample injections, this approach increases the sample throughput significantly.[616]

DNA fragments suitable for partial denaturing electrophoresis consist of two contiguous domains: a low-melting segment where a mutation is present, and a high-melting one (also called GC clamp) which stabilizes the structure and ensures it will melt only partially.[620] Such a high-melting domain can be a natural part of the fragment or easily be attached by use of one GC-rich primer of about 40 nt in the PCR amplification of the analyzed fragment. Another way to attach a clamp to any target of interest is through a ligation procedure.[621] The schematic melting profile of a two-domain fragment with the melting temperature of a base pair plotted against its position in the fragment is presented in Figure 18. The curve can be constructed theoretically based on a nucleotide sequence and the concentration of electrolytes and denaturants present in the solution.[622,623] In Figure 18 the low-melting domain starts to dissociate if the temperature increases over 65 °C. Most striking is the effect of base pairing mismatch on the melting temperature of a heteroduplex as indicated by the dotted curve. Under the optimum temperature, the dynamical equilibrium between the unmelted and partially melted forms, which is sensitive to any mutation present, determines their respective electrophoretic mobilities. As the temperature increases, the equilibrium shifts toward the partially melted form with a totally dissociated low-melting domain and the fragment mobility decreases. Subsequently, the separation selectivity of wild-type versus mutation-carrying fragments is also reduced. In the scheme presented in Figure 18, the high-melting domain starts to dissociate at a temperature over 85 °C and the fragments become totally denatured, dissociating into single strands. Under these conditions the fragments with point mutations cannot be separated.

A scheme of nine possible duplex combinations of strands coming from one wild type and two mutant homoduplexes together with the expected temperature trends of partial melting and migration time is presented in Figure 19. Each of the two mutant fragments carries one point mutation, the substitution of AT to GC pair, at different positions as indicated. The heteroduplexes are formed as random combinations of strands after fast cooling the mixture of completely dissociated fragments. The mutation-containing heteroduplexes dissociate more easily and in a more relaxed conformation migrate through the sieving medium slower than the homoduplexes. As a result, the homoduplexes and heteroduplexes containing different mutations can be separated from one another.

The separation mechanism must be viewed as a dynamical process. The more frequent the dissociated state of the low-melting domain, the slower the migration of the fragment through the sieving medium. This dynamical concept of migration of partially melted fragments is also supported by the fact that the width of their zones decreases with temperature and increases with electric field strength. Such behavior indicates the effect of a relatively slow dissociation kinetics.[624,625] The unmelted, double-stranded molecules migrate fastest, and their peaks are much narrower.

3.5.1. Constant Denaturant CE

The methodological and instrumentation principles of this particular CE technique combined with LIF detection were first described by the Thilly and Karger groups in 1994.[612]

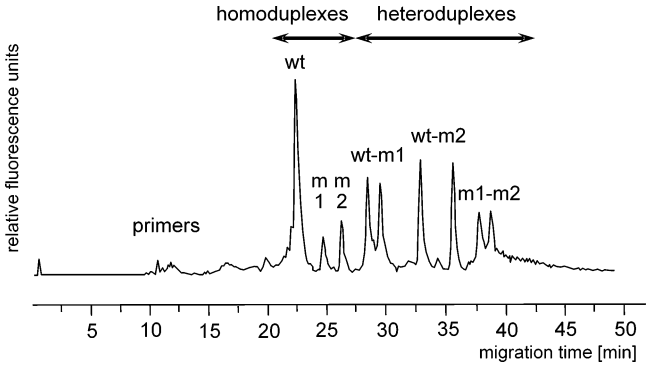


Figure 20. Separation of three homo- and six heteroduplexes shown in Figure 19. Fragments amplified from p53 gene. Wt = wild type DNA; m1, m2 = mutation substitutions A > G at different positions. Separation conditions: 14% solution of LPA (180 kDa) in 50 mM Tris−TAPS buffer at 79 °C; L_C = 55 cm; L_D = 40 cm; E = 360 V/cm. Unpublished authors' result obtained in cooperation with the laboratory of Prof. B. L. Karger, The Barnett Institute, Northeastern University, Boston, MA.

In this paper, the effect of electric field strength (83−250 V/cm), temperature (31−40 °C), and concentration of denaturing additives (3.3 M urea and 20% (v/v) formamide) on the migration of a model sample were studied. In later studies addition of denaturants was omitted and an elevated temperature was the only parameter found to control the partial denaturing.[624,626] It has been found that the above-mentioned phenomena of melting−reannealing kinetics play a crucial role in the separation efficiency, and therefore, high temperatures and optimum migration velocities are beneficial for resolving electrophoretic zones.[624] Another parameter which controls the kinetics is the counterion concentration. It is known that a higher cation concentration accelerates the renaturation. Thus, addition of 30 mM Na^+ as sodium borate to the conventional TBE buffer is used to reduce peak broadening in CDCE.[624]

An important variation in CDCE is the conversion of mutants into heteroduplexes with the wild-type sequence. By boiling a mixture of a mutant sample with a predominant wild-type sequence, mutant homoduplexes are converted to heteroduplexes containing one wild-type strand. Such heteroduplexes have significantly lower melting temperatures than wild-type homoduplex, and thus, mutations can be readily detected. An example of such an analysis is shown in Figure 20. Here, the separation of three homo- and six heteroduplex fragments amplified from p53 gene with two mutations at different positions (already described in Figure 19) demonstrates the capability of this method.

It has been estimated that in order to study some rare variants of mutations in human populations, sensitivity to detect one mutated molecule among 10^7 wild-type ones is required.[612] This number determines the total amount of DNA which must be injected into the capillary if a low-frequency mutation is to be detected. Since the detection limit of LIF in capillaries is 10^2-10^3 of fluorescently labeled DNA molecules, the total amount of DNA loaded into capillaries should be derived from more than 10^9 human cells to satisfy the sensitivity threshold.[627] This fact obviously represents serious limitations for the narrow bore capillaries routinely used in CE. Therefore, wide-bore capillaries of i.d. up to 540 μm have been tested. The capacity and sensitivity to detect point mutations was improved by 3 orders of magnitude. However, in order to keep the separation efficiency, the separation speed had to be reduced, which resulted in a 5-fold increase in analysis time.[627] The sensitivity demands are not so severe when mitochondrial point mutations are analyzed since their rate is 20 times higher than in nuclear DNA. Thus, an injection of 10^8 copies and detection limits better than 10^{-6} proved satisfactory for detection of mutations in human mitochondrial DNA in 75 μm i.d. capillary.[628,629]

Application of high-fidelity PCR is one prerequisite for improving the sensitivity. The errors which occur during DNA synthesis (PCR noise) introduce additional artificial mutations which can confound the mutation analysis in DNA diagnostics and screening.[630,631] It has been shown that with DNA polymerase, *Pfu*, the average error rate is decreased to 6.5×10^{-7}. This is a rate which is low enough for detection of point mutations in nuclear DNA.[630,632]

CDCE proved to be the most sensitive in the detection of mutations in the K-ras gene exon 1 when compared to either the allele-specific polymerase chain reaction or temporal temperature gradient electrophoresis.[633] Recently, a CDCE methodology was developed for automated analyses in commercial single-capillary[634−637] and capillary array[638,639] instruments with LIF detection. The most important advantage of CDCE is the ability to detect rare mutations in the pooled DNA samples of many individuals. Thus, the 1% detection limit of CDCE for variants in the epithelial sodium channel genes, which causes a severe form of hypertension (Liddle syndrome), indicates that up to 100 individuals can be analyzed in one CDCE run.[640] Other studies illustrate that the CDCE-based mutational spectrometry of DNA pools offers a feasible and cost-effective means of testing in order to define the fine structure map of genetic variation in large population samples. The cytotoxic T lymphocyte-associated antigen-4 genes in type 1 diabetes were scanned for unknown point mutations in pools of genomic DNA from a control population of 10 464 young American adults.[641] Also using CDCE the mutations of the human P-globin gene were scanned in a population of 5028 individuals as a single pooled DNA sample. Three point mutations were identified ranging in mutant frequency from 0.13 to 0.0005.[642]

A substantial innovation in mutation screening is represented by the automated multicapillary instrument HTMS (SpectruMedix LLC, State College, PA) for high-throughput mutation spectrometry furnished with a fraction collector.[367] The fraction collection module is a microwell plate fabricated from buffer-saturated agarose gel moving during the separation on a 3-axis translation stage.[365] The motivation for the preparative step is that the collected fractions can easily be subjected to further treatment including PCR, restriction analysis, sequencing, etc., to identify the detected mutation. The instrument features high optical sensitivity (detection limit of 10^{-12} M fluorescein) and automation for sample delivery, injection, matrix replacement, and fraction collection. The capillary array is divided into six groups of four capillaries, each of which can be independently set at a temperature up to 90 °C with a precision of ±0.01 °C by solid-state heaters. Detection of low-frequency alleles from pooled samples using CDCE demonstrates the capability of the system.[367]

3.5.2. Denaturing Gradient CE

DGCE developed by Righetti's group[18,613,625] is a capillary modification of gradient gel electrophoresis.[610,620,643−645] As outlined earlier, the denaturing gradients can be generated as either space or temporal variations of the denaturing agents

and temperature. In Righetti's experimental design the temporal temperature gradient of the electrolyte inside the capillary is generated internally simply by changing the running voltage applied at the capillary ends. Thus, the Joule heating due to a gradual increase in the electric current produces the temporal gradient of temperature which is evaluated theoretically by a computer program.[646,647] In temperature gradient capillary electrophoresis TGCE native fragments are injected into a capillary maintained at a temperature (typically about 60 °C) below the melting point of the low-melting domain and during the course of electrophoresis the temperature increases slowly (0.05−0.2 °C/min) above the melting temperature. Since the temperature ramp must be very shallow for good resolution, the absolute temperature increment is 1−1.5 °C. In order to generate this temperature gradient, the increase in voltage needed is typically over several kilovolts. The time-programmed temperature approach has been applied for detection of point mutations in the human genome.[613,648,649] Gelfi et al. used DGCE with temperature programming for detection of mutations in cystic fibrosis transmembrane conductance regulator gene[613,648] and β-thalassemia mutations in β-globin gene.[649]

Several papers focused on development of high-throughput automated instruments with precise control of spatial and temporal temperature gradients.[650] Yeung's group used two simple heating devices controlling a temperature gradient in separation capillaries for detection of unknown point mutations. A temporal gradient was established by the temperature programming of an aluminum heating plate connected to an array of 96 capillaries through a thermal conductive paste.[270] In another arrangement a concentric heat exchanger with counter or parallel flow of deionized water through an inner and outer jacket provided continuous spatial or temporal temperature gradients.[651] In both cases, differences between homoduplex and heteroduplex fragments were recognized in temperature gradients as high as 10 °C (60−70 °C) per analysis run or capillary length. A similar gradient (55 °C for 10 min, 55−65 °C for 25 min) generated in an adapted commercial Beckman P/ACE 5010 system enabled detection of the Pro102Leu mutation in the open reading frame of the prion gene associated with the Gerstmann−Sträussler−Scheinker disease.[652] Temperature gradients, which cover a broad range of melting temperatures, are advantageous for massive screening of unknown mutations. It follows from the previous discussion, however, that the separation selectivity and efficiency decreases with the breadth of the temperature ramp. Thus, it is always a trade off between the separation resolution and range of detectable mutations. The Spectrumedics HTS 9610 was developed for high-throughput mutation detection by TGCE followed by sequencing of chosen fragments. It is a 24-, 96-, 129-, or even a 386-capillary fully automated sequencer with on-column LIF detection. Temperature control of the capillary array is provided by hot air circulation with a precision of 0.1 °C. Typically, a temporal temperature gradient of 10 °C (60−70 °C for 21 min) at a step rate of 0.48 °C/min is used.[653,654] Under similar conditions, the sensitivity of detecting a heterozygote in pooled samples, modeled as the ratio of the amounts of mixed wild-type and mutant homozygous samples, was evaluated as being 1:20.[653] In the past few years, temperature gradient CE has been becoming increasingly popular in medical practice.[655−658]

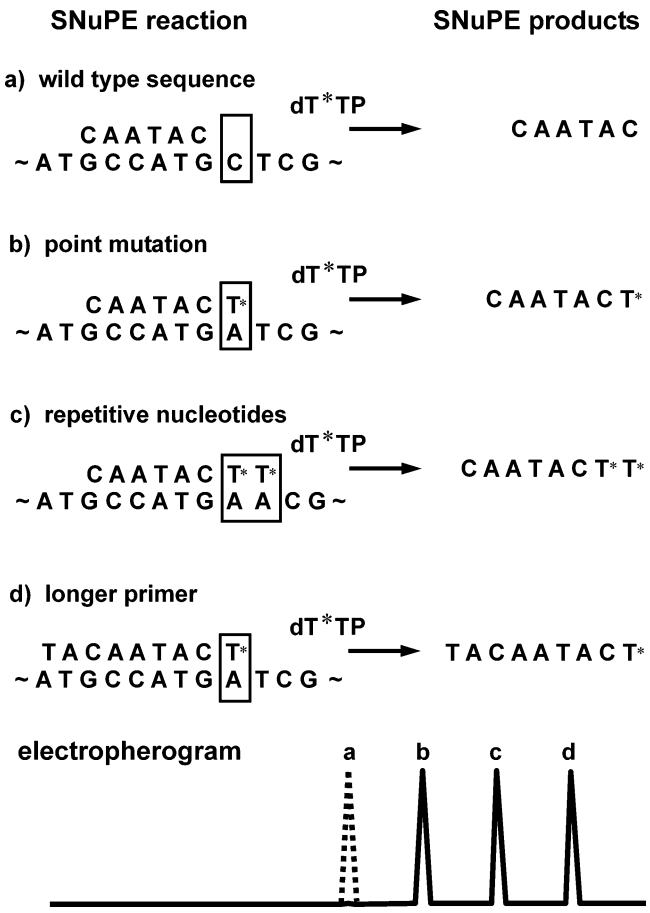


Figure 21. Scheme of SNuPE technique. Size-based separation reveals extension of primer: (a) no extension on wild-type template (invisible peak), (b) single nucleotide extension on expected point mutation, (c) extension of two nucleotides, (d) several mutations can be detected by multiplex reactions with primers of different lengths. The asterisk (*) marks the fluorescently labeled deoxy-thymidine triphosphate.

3.6. Single-Nucleotide Primer Extension

The single-nucleotide primer extension (SNuPE) method was developed for detection of known point mutations and SNPs.[659,660] Nowadays, two main approaches are implemented in clinical practice: analysis of SNuPE products by separation techniques or using microarrays.[661−663] SNuPE is a modification of the Sanger sequencing reaction, where only dNTPs or ddNTPs of a single base are present in the reaction mixture together with a template, oligonucleotide primer, and thermostable polymerase. Therefore, the term minisequencing (usually when primers or templates are immobilized on a solid surface) can also be found in the literature. Primers and dNTPs are chosen to be complementary either to the sequence immediately upstream of the mutation site or to a nucleotide at this site Thus, a DNA polymerase recognizes a nucleotide at the mutation site with a high specificity and dNTP extends the primer only if it is complementary. The advantage of this enzymatic reaction over hybridization with a specific probe is its high specificity, accuracy, and robustness. SNP tests can provide several extension products as shown in the schemes in Figure 21. Model reactions a and b represent the setup where dT*TPs (asterisk marks the fluorescently labeled deoxy-thymidine triphosphate) are added to the reaction mixture to detect the presence of the A > C substitution in a model sequence. Therefore, no primer extension occurs on the wild-type sequence

(reaction a), while the presence of adenine substitution leads to incorporation of labeled dT*TP into the primer (reaction b). If ddNTP terminators are present in the reaction mixture, either a single or no nucleotide is attached to the primer sequence. However, if dNTPs are used and there are two or more of the same nucleotides in a row at the mutation site on the template, then the respective number of the nucleotides will be attached to the primer sequence (reaction c). Using primers with variable sizes enables multiplexing of SNuPE reactions.[664] Thus, more than one point mutation in a single reaction can be detected at different positions on a template and the primers of different sizes provide a size-selective separation of products (reaction d). The electropherogram in Figure 21 shows a schematic separation of ssDNA products, usually performed in conventional denaturing electrolytes for DNA sequencing. Here, the invisible zone of unextended primer (reaction a) is followed by the products of reactions b−d.

Similar information can be obtained when a single fluorescently labeled primer is extended by more than one nucleotide in a mixture of a single ddNTP (complementary to a point mutation) and other three dNTPs. In this case the presence or absence of the mutation are indicated by fragments of specific sizes since the ddNTP blocks the extension either at the position of the substituent or at the subsequent occurrence of the same nucleotide.[665] This way, only a single primer extension reaction is satisfactory for identification of a heterozygote. Barta et al. used this strategy for detection of the most common mutation in the 21-hydroxylase gene.[666] The occurrence of the individual peaks of Cy5-labeled 19-mer primer in excess together with 26- and 35-mer products corresponds to this type of mutation. Thus, the presence of 19- and 35-mer, 19- and 26-mer, and 19-, 26-, and 35-mer indicates the wild-type template and homozygous and heterozygous mutations, respectively. The three fragments were separated in a 10% solution of PVP (mol mass 1.3 MDa) in 90 s.

Considerable effort has been invested to increase the multiplexing capabilities of SNuPE analyses. A SNP genotyping of multiple loci of a sample in a single reaction and single subsequent analysis is a prerequisite for high-throughput and cost-effective clinical screening. High-resolution CE with LIF detection provides several ways to increase the throughput. An increase in information outcomes from the analyses can be achieved not only by application of capillary arrays for multiple parallel analyses[113,653,667,668] but also by the selective size of primers[113,664,669,670] and four or more of fluorescent labels attached to dNTPs, ddNTPs, or primers.[113,665,667,671−674] The sizes of the primers indicate the position of the polymorphism, and the fluorescent tags can identify the polymorphic nucleotide at a site. To allow the control of optimum annealing conditions for each primer, individual reactions can be performed in separate tubes and pooled before the separation.[664] A significant sequence-dependent mobility shift of SNuPE products extended by different nucleotides has been demonstrated by Matyas et al. They separated products of the same size, synthesized on the genomes of heterozygous individuals extended by TAMRA-labeled ddCTP and ddUTP, respectively. (See Figure 22).[675]

Baron's group used free-solution separation (ELFSE), i.e., without a polymer sieving medium, in a 96-capillary system to demonstrate multiplexed SNP genotyping of several loci in a single reaction with a single subsequent analysis.[113]

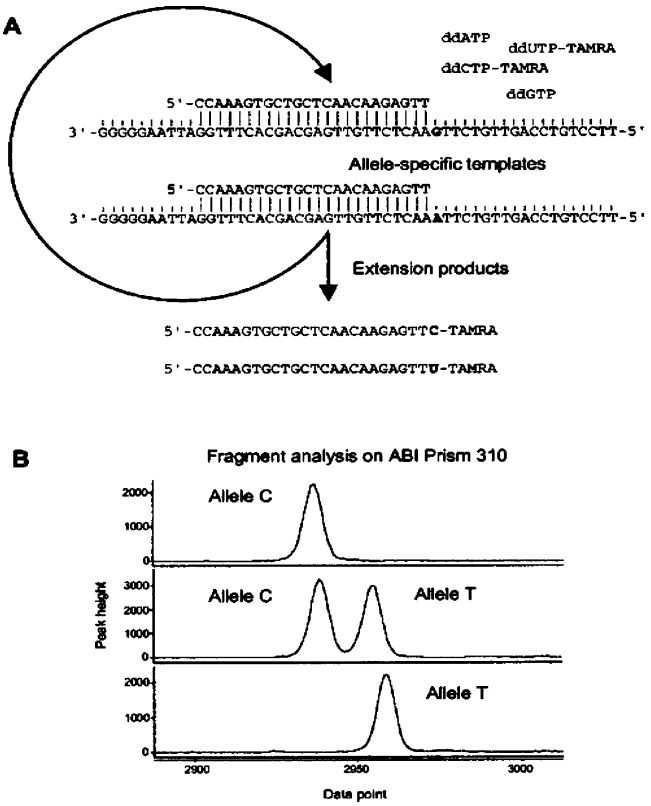


Figure 22. Schematic representation of the SNuPE assay exemplified by analysis of the *CYBB* 285C > T point mutation. (A) Depending on the allele, the SNuPE primer is extended by either ddCTP-TAMRA or ddUTP-TAMRA in a one-tube reaction. (B) The resulting allele-specific products are distinguished and identified on the basis of sequence-dependent mobility in LIF-CE system. Reprinted with permission from ref 675. Copyright 2002 Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

Three unique oligonucleotide primers designed as probes for mutations of clinical importance in the human p53 gene were covalently attached to three unique frictional end labels, poly(*N*-methoxyethyl)glycine oligomers of 10, 20, and 30 monomers in length. The short DNA molecules elongated by an uncharged polymer chain do not behave like homogeneous polyelectrolytes, their charge to size ratios differs, and therefore can be separated in free solution. The advantage of this technique is a fast separation of SNuPE products in free denaturing electrolyte, which can easily be performed in microfluidic devices.

The end-column electrochemical detection of SNuPE products was described as an alternative to LIF detection.[431] A 32 μm carbon fiber inserted into the anodic end of the separation capillary plays the role of a working electrode for the sinusoidal voltametry[430] of DNA fragments labeled by ferrocene acetate. The electroactive molecule is covalently attached to the 5′ end of a primer[431] or to a ddNTP terminator.[433] The idea behind the implementation of electrochemical detection is to construct a miniaturized, relatively inexpensive and portable instrumentation for clinical practice. Subsequently, this electrochemical detection system was tested for SNuPE analysis in a microfluidic format.[434]

4. Applications

Various DNA diagnostic methods have very important applications not only in the field of clinical chemistry but also in genetic and medical research as well as in forensic

science. In recent years, an increasing number of papers have focused on the development of CE methodologies for both detection of known mutations implicated in human diseases and discovery of new ones.[16,59,581,676−680] Mutations in both coding and noncoding regions of genomic DNA lead to multiple hereditary diseases including metabolic disorders and neuropathological diseases. They are also implied in a vast array of disorders such as cancer, heart diseases, atopic disease, autoimmunity diseases, etc. Molecular identification of pathological microorganism based on DNA analysis is another important example of the use of DNA diagnostics in clinical medicine.[212,277,530] The potential of CE techniques for haplotyping, an essential method for the association-based gene mapping of disease-susceptibility genes in the study of Mendelian and complex diseases, was also demonstrated.[482,681]

4.1. Cystic Fibrosis

Cystic fibrosis is a monogenic, chronic disease affecting multiple organ systems, leading to death with an average life span of 28 years. It is one of the most common autosomal recessive disorders found in Caucasians in Europe and North America with an incidence estimated at 1 in 3000 live births. Detection of mutations causing cystic fibrosis (CF) has been the object of tremendous research efforts over many years. Linkage analysis indicates a presence of a single CF locus on the long arm of the human chromosome 7, band q 31−32. The locus spans approximately 250 kbp, contains 24 exons with a total length of 6129 bp, and encodes a polypeptide of 1480 amino acids entitled the cystic fibrosis transmembrane regulator (CFTR). A 3 base pair (CTT) deletion is the most common mutation in the CF gene and results in the absence of the phenylalanine codon at position 508 of the amino acid sequence. This mutation has been designated ΔF508 and is thought to affect a putative ATP binding domain of CFTR. The commonly used primers for a PCR assay for detecting the ΔF508 mutation provide fragments of sizes 95 (mutant) and 98 bp (wild type). Such fragments differing by 3 bp can easily be resolved using electrophoretic techniques (see Figure 23). Fast and easy analysis using capillary electrophoresis is especially suitable for rapid detection of ΔF508 in "urgent samples" in prenatal diagnostics, thus permitting a low-cost screening of the population.[62,682−686]

More than 1500 CFTR mutations are listed in the CF Mutation Database. Gelfi et al. used various CE techniques for detection of many of the frequent CF mutations.[613,625,683,684,687,688] They analyzed point mutations located in exon 11 (G542X and others), exon 17b (R1066H, R 1066C, F1052V), exon 20 (S1251N), and two polymorphisms located in exon 14a (V868V, T854T) of the CFTR gene using DGCE with temperature programming.[613,625] Sequence variation is sometimes designated as polymorphism, indicating that it is not in fact disease causing. The same group identified two main allelic forms, one hexameric and one heptameric of a tetranucleotide (GATT) repeat polymorphism at the junction of intron IVS6a and exon 6b.[625,687] The hexameric allele has been found to be strongly linked to the ΔF508 mutation and, in combination with additional polymorphic markers the GATT repeat, may allow determination of the chromosomal background from which ΔF508 arose. The poly-T tract in intron 8 of the CFTR gene was identified in three variants 5T, 7T, and 9T (5T generates anomalous protein) and analyzed in isoelectric 200 mmol/L

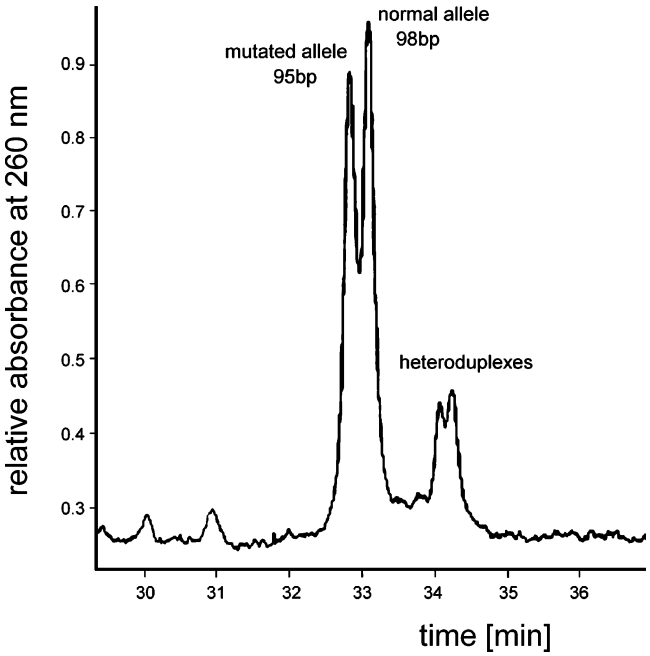


Figure 23. Detection of the most common mutation ΔF508, a 3-base pair (CTT) deletion, in cystic fibrosis transmembrane regulator (*CFTR*) gene of a heterozygote. AFLP method provides fragments of sizes 95 (mutant) and 98 bp (wild type) and their combinations in the form of heteroduplexes. Electrophoresis at 164 V/cm in a 50 cm long fused silica capillary filled with 2% solution of SeaPrep agarose in 1xTBE buffer. Unpublished authors' results.

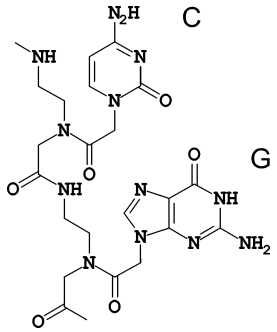


Figure 24. Segment of peptide nucleic acid.

histidine buffer (pH = pI = 7.6) in 10% polyacrylamide. The combination of very low conductivity together with a satisfactory buffering capacity allowed separations in less than 10 min.[688]

An interesting approach to mutation detection is the use of labeled peptide nucleic acid (PNA) oligomers as hybridization probes.[689,690] The structure of a PNA chain segment carrying cytidine and guanine bases is presented in Figure 24. PNA oligomers have a higher affinity and selectivity in binding DNA and RNA than the naturally occurring nucleotides. This permits specific sequence detection with simultaneous size separation of the target DNA. Perry-O'Keefe et al. tested this method for detection of a single base mutation in a clinically relevant assay. They detected a very frequent *W1282X* mutation, a substitution of G for A at position 4041 in exon 20 of the CFTR gene of cystic fibrosis carriers.[691] First, a fluorescein-labeled PNA probe was hybridized to a denatured DNA sample at low ionic strength. The probes which carried 15 bases were synthesized for both mutant and wild-type sequence. Then the sample was injected into the capillary filled with a solution of 1% PEO in 1xTBE buffer, and the PNA/DNA complex was separated from any

excess of the PNA probe at a temperature of 50 °C. The elevated temperature increased the stringency of the method. The only charge of the neutral PNA backbone is that of a fluorophore. In principle, the analysis can be performed also in free denaturing electrolyte, i.e., without a sieving medium.

4.2. Down Syndrome

The good quantitation possibilities of CE can also be demonstrated in gene dosage analysis in prenatal diagnosis of Down's syndrome. Down's syndrome is caused by partial or complete trisomy of chromosome 21. The hallmark of this disease is mental retardation and cardiac and other malformations. Detection of chromosome 21 dosage in fetal cells obtained from amniotic fluid or neonatal blood allow for prenatal or postnatal diagnosis of trisomy 21, respectively. It is clear that the initial step of PCR amplification has to be conducted in a quantitative manner to ensure that the amount of product is proportional to the amount of the original template.[692]

Furthermore, the injection and detection procedures in the CE system should not discriminate any of the analyzed DNA fragments; otherwise, the extent of the discrimination must be known.[693,694] The method was developed for a highly polymorphic tetranucleotide STR region of the chromosome 21, the specific D21S11 marker.[695] Since the polymorphism serves only as a marker which does not cause the disease, the following quantitative results can be expected. (1) For normal homo- or heterozygotic individuals, a single peak with double intensity or two peaks with equal intensity will be present, respectively. (2) For individuals affected by trisomy 21, three variants are possible: (i) three peaks with equal intensities, (ii) two peaks, one with double and one with single intensity, and (iii) a single peak of triple intensity in rare cases of homozygosity.

4.3. Marfan and Ehlers–Danlos Syndrome

Marfan and Ehlers−Danlos syndromes are genetic disorders resulting in defects in connective tissue formation and subsequent defects of skin, joints, bones, eyes, and aorta. The former is caused by a mutation in the fibrillin gene *FBN1* and the latter by defects in genes encoding various types of collagen (*COL* family). Another example of a quantitative CE analysis in DNA diagnostics is the quantified analysis of SNPs as genetic markers for these diseases. SNPs are increasingly popular as genetic markers because of their great abundance and amenability to fully automated genotyping. As already shown, SNuPE technology in connection with fluorescently labeled dideoxy terminators has numerous advantages for detection of SNPs using CE. Matyas et al. used a CE-LIF system for an accurate quantification of SNP variants in transcripts and pooled DNA in a one-tube SNuPE reaction to determine the transcript levels of the heterozygous human genes *FBN1* and *COL5A1*.[675] The *FBN1* gene on chromosome 15 encodes fibrillin, while the *COL5A1* on chromosome 9 encodes type V collagen. Fragments of four different genes (*FBN1*, *COL5A1*, *CYBB*, and *HBB*) containing seven different SNPs representing three different base changes (C > T, A > G, and A > T), were chosen to validate the methodology. The SNuPE reaction products labeled with TAMRA fluorescent dye were separated in a POP-4 separation medium. Interestingly, all product pairs, both mutated and wild type, 20−25 nucleotides in size, differing only in their 3′ extended base composition and labeled with the identical TAMRA dye were completely separated (see Figure 22). Thus, not only the presence of different alleles but also their relative frequencies could be determined both reproducibly and accurately. It has been shown that a difference in allele frequencies as low as 0.8% and relative allele frequencies as low as 1% can be accurately detected by this method.[675]

4.4. Thalassemia

The thalassemia diseases are a group of inherited anemias characterized by a reduced production of hemoglobin. The most common type of adult hemoglobin, hemoglobin A, is composed of four heme groups, two α-globin chains and two β-globin chains. There are two α-globin genes on each chromosome 16 and one β-globin gene on chromosome 11. The α- or β-thalassemias are characterized by deficient production of the respective α- and β-globin polypeptide chains. There are hundreds of mutations within the β-globin gene, but approximately 20 different alleles represent 80% of the mutations described worldwide. Gelfi et al. detected three point mutations and one deletion in β-globin gene using DGCE.[649] The same group developed an interesting method for separation of relatively short fragments of globin-encoding DNA in free solution acidic buffers.[696] The four different nucleotides have significantly different charges at pHs lower than 3.5. Thus, this technique allows electrophoretic separation of fragments shorter than 100 bases differing even by a single nucleotide in an electrolyte without addition of a sieving medium. In this study the point mutation β-39 (C > T) in codon 39 of β-globin gene was analyzed. At pH 3 in 50 mM of phosphate buffer with 7 M urea the C base is protonated while T does not carry any charge. Thus, the difference in charges of the mutant and wild-type ss fragments is sufficient for the baseline separation in the free solution.[696]

Multiplex minisequencing with site-specific primers and fluorescently labeled dideoxynuxcleotides was successfully used to detect several common β-thalassemia mutations. Mini-sequenced products were separated and detected by capillary electrophoresis followed by automated genotyping.[673]

4.5. Duchenne and Becker Muscular Dystrophies

Numerous research groups have focused their efforts on the detection of mutations involved in hereditary muscular dystrophies, e.g., Duchenne and Becker muscular dystrophies (DMD/BMD). These genetic disorders are characterized by progressive muscle dystrophy and weakness that frequently lead to complete immobility and, subsequently, death due to respiratory infections. Molecular genetic mapping studies indicate that both diseases are X-linked disorders caused by mutations in the remarkably large gene, spanning 2.5 megabases at *Xp21*. This gene encodes a protein of 427 kDa called dystrophin. Duchenne dystrophy affects approximately 3 in 10 000 live-born males, while Becker dystrophy is about 10 times less frequent. Approximately two-thirds of the mutations responsible for these two disorders are deletions of one or many exons in the dystrophin gene. About 30% of cases of Duchenne dystrophy are mutations de novo, and thus, 70% of mothers of affected children are carriers of the mutation. In the remaining 30% of cases the disease results from a new mutation. Therefore, a fast and relatively simple screening method for this mutation would be highly desir-

able.[211] Gelfi et al. optimized the molecular mass of a sieving polyacrylamide and composition of PCR primers for multiplex amplification of fragments related to frequent deletion sites in 18 exons of the dystrophin gene. The 18 fragments (sizes 88, 113, 139, 154, 170, 196, 202, 238, 268, 271, 313, 331, 357, 360, 388, 410, 506, and 547 bp) were amplified and successfully separated in a 6% solution of LPA (mol mass 250 kDa[697]) in 1×TBE buffer (pH 8.3).[539]

Characterization of carrier status in females as well as exon duplications or deletions in affected males can be achieved using various precise quantitative approaches for gene dosage analysis. It should be emphasized here that a simple quantitative estimation of a component from the respective peak height or by a simple visual inspection is inaccurate in the majority of instances. In any quantitative analysis the relationship between the sample amount and the detection signal must be known. The area of a peak in CE-LIF is determined not only by the amount of the sample injected but also by its migration velocity and the intensity of the fluorescence. Thus, the recorded peak areas must be corrected. Fortina et al. reported the use of a quantitative CE-LIF analysis of multiplex PCR products for determination of carrier status of DMD/BMD. The authors used a 0.5% solution of HPMC in 1×TBE buffer with addition of 0.5% glycerol and an intercalating fluorescent dye YO-PRO-1 for separation of the mixed products from multiplexed PCR reactions. All products ranging in size from 113 to 547 bp were separated in a mere 10 min.[541] Obviously, the PCR must be truly quantitative, i.e., all fragments must be amplified with the same yield. In the case of X-linked disorders such as DMD, there are only three possible quantities for the fragments amplified on particular exons: (i) double the amount of the product amplified on the gene of a healthy woman or an affected man with an exon duplication, (ii) an amount equal to a male or a carrier heterozygotic woman, or (iii) the absence of the respective fragment in the case of a proband. Therefore, only a relative correction method using a standard fragment (756 bp) was used.[541]

Several reports showed an increased throughput in the detection of mutations causing DMD/BMD using a CAE system with a bundle of 48 capillaries[535] and ultrashort migration distances in microfluidic devices.[209,698,699] An example of such fast separations is shown in Figure 25.

Cheng et al. demonstrated the feasibility of performing complex PCR assays in microfabricated devices.[698] An effective method for amplification of the loci contributing to DMD/BMD was performed on a silicon−glass chip and transferred to another microfluidic device for electrophoretic analysis. The micromachined device used in this study had channel widths of 50−60 μm and a depth of 10 μm; sample injection was by channel cross, and the separation distance was 2.5 cm. The channels were filled with an in situ polymerized separation matrix, 3% (w/v) LPA in 0.5×TBE with addition of a 1 mM TO-PRO intercalating fluorescent dye. Migrating DNA fragments were excited by the 514 nm line of an argon-ion laser, and the fluorescence signal was collected using a 20× objective lens (NA = 0.42), followed by spatial and spectral filtering.[698] It is worth mentioning here that microfluidic devices have the advantage of eliminating the electrophoretic injection bias frequent in CE since the samples of DNA fragments are introduced hydrodynamically via the classical channel cross. This bias deteriorates quantitative analysis due to fragment size discrimination when electromigration sampling is applied.[693,694]

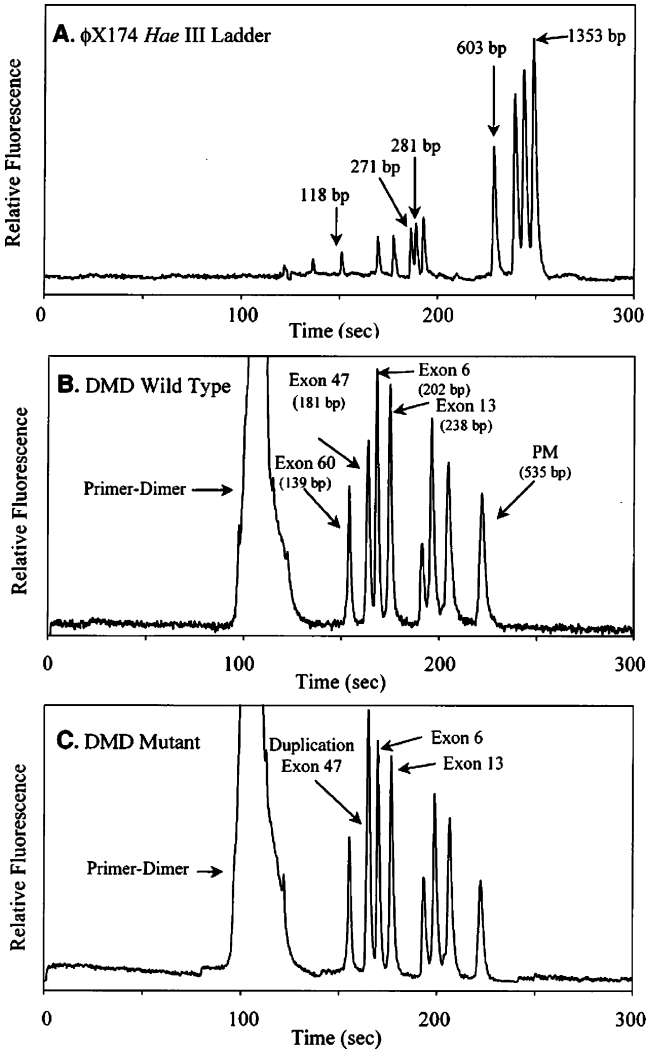


Figure 25. Microchip separations employing the optimized buffer system. (A) Separation of DNA ladder with 3.5% HPC, 80 mM MES/40 mM TRIS buffer system with a field strength of 375 V/cm. (B) Separation of a mulitplex PCR sample from a patient negative for DMD utilizing the optimized buffer system. Field strength of 375 V/cm. (C) Separation of multiplex PCR sample of a patient diagnosed with DMD utilizing the optimized buffer system. Field strength of 375 V/cm. Reprinted with permission from ref 209. Copyright 2003 American Chemical Society.

Ferrance et al. used commercially available microchip electrophoresis instrumentation and methodology[700] for the analysis of DMD-associated mutations in less than 2 min.[699] Automatically calculated peak areas of normal PCR- amplified fragments were compared to those amplified using genomes with mutations. It is known that not all DNA targets amplify to the same extent, and concentrations of amplified products may vary between reactions. Thus, multiplex PCR amplification may introduce an error in quantification. Therefore, the authors reduced the number of PCR cycles to less than 20 in order to obtain more accurate quantification.[699]

4.6. Leber's Hereditary Optic Neuropathy

This disease is caused by three types of mutations affecting genes coding for the protein involved in mitochondrial electron transport. Clinically, the disorder is characterized by apoptotic cell death of the retinal ganglion and subsequent optic nerve atrophy. Affected individuals suffer from pro-

gressive vision loss frequently to the point of complete blindness. There is no known therapeutic approach for stopping or reversing the process. The three point mutations (G > A) responsible for the disease in the mitochondrial genome at positions 3460, 11 778, and 14 459 were successfully analyzed using the ligase chain reaction (LCR)[540] and SNuPE[664] products. Muth et al. optimized the temperature and number of cycles of LCR as well as the separation conditions for a fast CE analysis with LIF detection. Three fragments carrying the mutations were amplified by multiplex PCR on the mitochondrial DNA template followed by LCR. The primers designed for LCR at positions 3460 and 14459 had additional polythymidine tails of 10 and 20 bases, respectively. Thus, the length differences of the LCR fragments were modified for easier separation. The fragments intercalated by ethidium bromide were analyzed in the capillary of an effective length of 7.5 cm filled with a solution of 1% methylcellulose in only 90 s.[540] Piggee et al. demonstrated the use of the SNuPE method for detection of the same set of mutations. They also amplified three DNA fragments from three mitochondrial DNA samples for use as templates in the SNuPE reactions. Fluorescently labeled dideoxy adenosine or guanosine was used as a terminator for the extension, making it possible to distinguish between the wild-type DNA (containing cytosine) and the mutant one (containing thymine). The products were separated in the capillary filled with a solution of 10% polyvinylpyrrolidone in 50 mM Tris-TAPS buffer and 3.5 M urea.[664]

4.7. Cancer

In contrast to hereditary disorders, which are either inherited or occur de novo, almost all cancers result from multiple mutations accumulated in an individual's genetic material later in life. DNA diagnostic methods are used routinely to detect residual malignant cells (indicating residual disease) after chemotherapy, whole body irradiation, or stem cell transfer therapy. The following DNA analysis techniques are frequently used for detection of mutations causing cancer:[479,701] RFLP/AFLP,[702−707] SSCP,[568,569,575,578,579,589,592,599,604,708−712] CDCE,[364,627,633−635,637,638,713−715] TGCE,[616,716,717] SnuPE,[584,675,718,719] and heteroduplex analysis.[272,705,714,720−722] Kuypers et al. developed a method for quantification of residual disease in patients undergoing treatment for follicular lymphomas.[723] In 70−90% of patients with this type of lymphoma a reciprocal translocation between chromosomes 14 and 18 is found and serves as a tumor-specific marker. This very common tumorigenic translocation is a reciprocal chromosome exchange that places the *bcl*-2 proto-oncogene (located on chromosome 18) under aberrant transcriptional control of the promoter for the immunoglobulin heavy chain gene (located on chromosome 14). Thus, PCR amplification of the region containing the translocation breakpoint can sensitively detect the presence of any malignant cells. The amount of cells can be estimated by a comparison of the peak areas of two DNA fragments with different sizes amplified using both the patient's and the standard genomes simultaneously. This competitive PCR amplification effectively eliminates the usual problems with variation of the product yield due to small changes in reaction conditions and provides a reliable quantification. Obviously, standards of related fragments and calibration are needed for evaluation of the absolute amount of molecules with the translocation.

In several publications, the variability in STR identified by CE was demonstrated as being a significant diagnostic marker of cancer.[724−730] Mathies' group developed a sensitive two-color labeling method with ET primers for the high-throughput STR-based screening of bladder cancer patients.[731] Detection of a size difference in PCR amplicons from normal and tumor DNA is an indication of expansion or deletion in the microsatellites in the five different loci used as markers. Normal and tumor DNA were labeled using ET primers with 5-carbohydroxyrhodamine-6G (R6G) and ROX as acceptors, respectively. (See Figure 7 for the structure of the ET primer.) The labeled fragments were separated in capillary arrays filled with a solution of 2% HEC with 1×TBE, 5.6 M urea, and 32% formamide. The variations in the ratios of the integrated peak areas of individual alleles (tumor/normal) served as criteria for the tumor diagnostics.

A widely used strategy for the preparation of fragments from coding regions of genomic DNA utilizes reverse transcription of mRNA to cDNA and its subsequent PCR amplification (RT-PCR).[706,732−740] Another method used in cancer diagnostics is the monitoring of telomerase activity, a useful biomarker for early detection of cancer. Telomerase activity correlates with tumor progression, indicating that tumors expressing this enzyme exhibit aggressive clinical behavior and unlimited proliferation and immortalization of cells.[274,741−743] Many other useful applications of CE in cancer research[274,657,744−747] and clinical diagnostics[658,721,732,734,738,748−751] have been published including the implementation of microfluidic devices.[271,710,720,737,742]

4.8. Forensic Applications

The speed and reproducibility of CE analysis makes it especially useful in forensic applications. DNA typing based on recombinant DNA technologies has become one of the most powerful tools in forensic medicine and criminal investigations including personal identification and determination of paternity.[680,752,753] Nowadays, PCR-based technologies detecting short polymorphic stretches of DNA are becoming increasingly utilized to replace classical fingerprinting. The DNA loci containing suitable polymorphism sites are situated exclusively in noncoding regions, which in fact represent about 90% of the human genome.[488] The targets for the classical VNTR are loci of up to 10 kb long and composed of segments of hundreds of nucleotides and can have more than one hundred repeats. However, analysis of polymorphism in VNTR typically requires 5−10 μg of intact genomic DNA, an amount that is frequently difficult to obtain.[680] Forensic DNA typing, therefore, often requires use of techniques that allow the use of much smaller samples and detection of much shorter repetitive loci. Such loci proved to be STR, which are composed of 2−7 nucleotides repeated up to a length of 80−400 bp. Due to the short segment of STR loci, the PCR of a single STR often requires as little as 50 pg of template DNA.[488,680,754]

As we know from Mendelian genetics, for any given gene one allele is inherited from the mother and one from the father. Thus, the comparison of about five single-locus DNA profiles produces strong evidence for identity and establishing paternity/maternity. In DNA typing, the identity is determined by calculating the probability that a given individual would have a specific set of alleles at a given STR locus. The multiplex STR methods currently used have matching probabilities so high that they can easily identify one single individual among all humans with practically no chance of an error. When 13 STR loci are analyzed and combined the

probability that two individuals would have identical sets is lower than one in a trillion.[755]

One of the first publications demonstrating the applicability of CE in forensic analyses was that of McCord et al. They analyzed two genetic VNTR loci D1S80 (human chromosome 1 locus 80) with a 16 bp repeat unit and the STR locus SE33 (ACTBP2). Their experimental design used a CE system with an absorbance detector and capillary filled with a replaceable 0.5% solution of HEC.[193] Srinivasan et al. described the analysis of three different genetic loci, VNTR locus D1S80, VNTR locus in the apolipoprotein B gene (14 bp repeat), and a 2 bp repeat in mitochondrial DNA, using DNA extracted from three human hair roots as a template.[387] While the initial effort to analyze these products using a CE system with UV detection at 260 nm failed, LIF detection of the products intercalated with TOTO and YOYO fluorescent dyes was successful.[387] Later on a genetic typing with the D1S80 allelic ladder as an absolute DNA standard was demonstrated in CE systems with LIF detection. Zhang et al. used a 2% solution of poly(ethylene oxide) (8 MDa) as a sieving medium and ethidium bromide as an intercalating fluorescent label.[260] Mitchel et al. used a 0.3% HEC and a YO-PRO-1 intercalating dye for analysis of the same locus.[392] The analyses of STR polymorphism of HUMTH01 allelic ladders with the four-nucleotide repeat unit AATG are very frequent. This specific marker is located within the intron 1 of the Tyrosine Hydroxylase (TH) gene on human chromosome 11. Gene environment interactions and sex-specific genetic effects have been observed when HUMTH01 variability was analyzed. McCord et al. analyzed genetic markers HUMTH01, vWA, and MBP by separating individual alleles in a 1% solution of HEC and detecting LIF signal of the DNA fragments intercalated by YO-PRO-1 fluorescent dye.[391] The STR locus vWA is a four bp repeat (AGAT) located on chromosome 12 within the intron 40 of the VonWillenbrand factor gene. The MBP is a human myelin basic protein gene on chromosome 18 which contains two STR, each of four bp repeat TGGA. There are other highly polymorphic loci of four bp STRs that are frequently used as genetic markers for personal identification. The mixtures of their alleles are also frequently used as the absolute sizing standards: HUMFES (AAAT), TPOX (AATG), CSF1PO (AGAT) and D5S818 (AGAT), D13S317 and D7S820 (GATA), FGA (TTTC). Although the polymorphic nature of these loci is characterized by four bp repeats, sometimes, due to a difference in length and sequence of the whole repeat element, even a single bp resolution is required for the proper characterization of an allele. For example, the TH01 allele containing nine complete tandem repeats (AATG) and a unit with only three bp (AAT) will differ by a single bp from the allele with 10 complete tandem repeats. Therefore, a level of a single bp resolution for fragments up to 400 bp in size is necessary for a reliable analysis of STR polymorphism.

Van der Schans et al. demonstrated that the separation of STRs is more selective when performed under denaturing conditions. They used the STR allelic ladders HUMTH01 and HUMFES and separated them in a solution of 4% polyacrylamide (polymerized at 4 °C) in 7 M urea and 50 mM TBE buffer.[560] Similarly, Zhang et al. reported the simultaneous analysis of vWA, TH01, TPOX, and CSF1PO STR loci under denaturing conditions with single-nucleotide resolution. The samples were separated in a denaturing mixture of 1.6% (8 MDa) and 1.5% (600 kDa) solution of PEO in 1×TBE buffer with 3.5 M urea.[756] Critical for this type of analysis is that the sample is completely denatured prior to analysis. Therefore, addition of formamide, heating to 95 °C, and a low ionic strength of the sample solution are recommended to protect its denatured state during injection. As an alternative, separation of the sample mixture has also been demonstrated in a similar sieving environment but without urea and using an elevated temperature program (70 °C start, 55 °C end). The high temperature not only increased the separation speed but also improved the peak shape.[756] A similar mixture of specifically fluorescently labeled allelic ladders was used for optimization of denaturing electrophoresis with HEC as a sieving medium using a highly automated single-capillary ABI Prism 310 Genetic Analyzer (ABI Foster City, CA) with multiwavelength fluorescence detection. The optimum separation of multiple loci (D3S1358, FGA, CSF1PO, TPOX, TH01 vWA, vWA) was demonstrated in 3% w/v HEC (40 kDa) with 100 mM Tris-borate (pH 8.4) and 7.1 M urea at a temperature of 60 °C within 30 min. The average resolution obtained was 1.4 bases for a 200 bp fragment with a standard deviation of sizing of 0.2 bases.[203] Sizing precision with a standard deviation ≤0.16 nt was achieved using denaturing POP-4 polymer consisting of linear poly(dimethylacrylamide), 8 M urea, 5% 2-pyrrolidone, and 1 mM EDTA.[538] The standard allelic ladders were resolved using this approach at a temperature of 60 °C. Implementation of multicapillary sequencers into gene typing opened up the possibility of fully automated analyses of many samples and simultaneous analyses of several specifically labeled loci in a single run. Reliable simultaneous genotyping of many samples is facilitated by the co-amplification of two or more polymorphic loci into one PCR. The sizing of over 240 samples of multiplex STR systems using capillary arrays yielded an average within-run precision of ±0.13 bp and between-run precision of ±0.21 bp for fragments up to 350 bp.[537] Up to 96 samples can be processed using capillary arrays filled with a solution of 1.25% HEC (150 kDa) in 1×TBE with 7 M urea and 10% formamide within 70 min.[537] Different multiplex STR systems can be analyzed simultaneously using capillary arrays as well as by utilizing the four-color detection system.[509] All of these innovations permit increased sample throughput. Significant progress in the development of electrophoretic microdevices is also having a strong positive impact on DNA-typing technology. For example, Schmalzing et al. simultaneously analyzed four STR loci on a 2.6 cm long chip in a mere 45 s (see Figure 26).[333]

5. Concluding Remarks

Over the past two decades, analytical chemists adopted the challenge of the Human Genome Initiative and successfully developed the CE instrumentation and methodology resulting in fully automated apparatuses capable of sequencing the human genome at a reasonable price. Not only DNA sequencing but also DNA genotyping based on multiplex analyses of polymorphisms and mutations have greatly benefited from these developments. Such analyses play an increasingly important role in biological and medical research as well as in clinical practice. The result is that CE fully automated systems have become the most widespread instrumentation for DNA analyses. There were four crucial developments in the evolution of these high-throughput automated systems: (i) powerful replaceable sieving media, (ii) arrays of more than 100 capillaries, (iii) highly sensitive

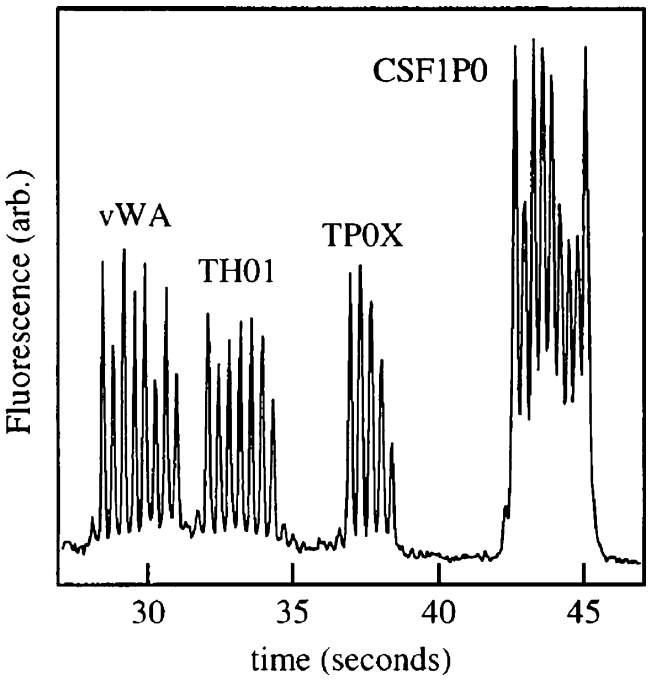


Figure 26. Microchip electropherogram for the four-locus *CTTv* allelic sizing standard. The chip contained 45-mm-deep channels, a 100-mm sample injector, and a 26-mm-long separation channel. The separation was performed at 50 °C with a field strength of 500 V/cm in a sieving matrix that consisted of 4% linear polyacrylamide in 1xTBE buffer with 3.5 M urea and 30% (vol/vol) formamide. Reprinted with permission from ref 333. Copyright 1997 National Academy of Sciences, U.S.A.

LIF detection systems compatible with capillary arrays, and (iv) sophisticated software enabling data processing within analyses times. This review has demonstrated the fundamental importance of miniaturization for development of high-throughput analytical technologies in this field. Further miniaturization of CE analytical instrumentation resulted in separations performed using microfluidic devices and decreased typical analyses times from minutes to seconds. Such instrumentation has been successfully commercialized, and nowadays, a few micromachined microfluidic devices are available on the market. The most noteworthy development, however, is research into single-molecule detection. Once, a method is capable of detecting a single particle or molecule, in principle, there is no need for their separation from both a quantitative and a qualitative point of view. Similarly, just like cells in flow cytometry, DNA fragments can be counted and sized as they pass a detector or collectively traced in the displayed fields.[757] Such analyses do not require any amplification of the analyte (very dilute solutions are preferable), any selective separation medium, efficient injection of a sharp zone, etc. The tendency to manipulate and detect single molecules will lead to instrumentation where microfluidic elements will serve merely as auxiliary equipment for the structural elements machined using nanotechnologies. It can be expected that such miniaturized devices for detection of single DNA molecules immobilized on defined surfaces or moving in a free electrolyte will prove to be efficient alternatives to CE in the future. According to recent estimates the sequencing rate of these innovative techniques could reach up to 125 million bases per hour, which is a 1000-fold increase in throughput over the current CE instrumentation using Sanger sequencing technology.[758]

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