Separation of DNA by Capillary Electrophoresis
Separation of DNA by Capillary Electrophoresis

Herb Schwartz\textsuperscript{1}

and

Andras Guttman\textsuperscript{2}

\textsuperscript{1} Palomar Analytical Services, 150 Montalvo Road, Redwood City, CA 94062
tel: (415) 365-3711; e-mail: heschwartz@aol.com

\textsuperscript{2} Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92634
tel: (714) 773-8211; e-mail: aguttman@ccgate.dp.beckman.com
# Table of Contents

About the Authors ........................................................................................................ v
Acknowledgments .......................................................................................................... v
Front Cover ....................................................................................................................... vi
Acronyms Used ............................................................................................................. vii

1 Introduction ................................................................................................................... 1

2 Fundamentals of Capillary Electrophoresis (CE) ..................................................... 4
   2.1 CE Instrumentation ......................................................................................... 4
   2.2 CE Modes ........................................................................................................ 6
   2.3 Theory .............................................................................................................. 9
       2.3.1 Migration Velocity ............................................................................. 9
       2.3.2 Efficiency and Resolution ............................................................... 11

3 Separation of DNA by Techniques Other Than CE ............................................. 13
   3.1 HPLC ............................................................................................................ 13
   3.2 Gel Electrophoresis: Polyacrylamide (PA) and Agarose ............................ 15
       3.2.1 Instrumentation and Detection ....................................................... 15
       3.2.2 Separation Mechanism .................................................................... 17
       3.2.3 Polyacrylamide Gel Electrophoresis (PAGE) .............................. 19
       3.2.4 Agarose Gel Electrophoresis ............................................................ 19
       3.2.5 Pulsed-Field Gel Electrophoresis (PFGE) .................................... 19

4 CE Methods: Principles and Strategies ............................................................... 21
   4.1 Free Solution Methods .................................................................................. 21
       4.1.1 CZE with Untreated or Coated Capillaries ...................................... 21
       4.1.2 CZE with Sample Stacking or ITP Preconcentration .................. 23
       4.1.3 Micellar Electrokinetic Capillary Chromatography (MECC) ......... 24
   4.2 Capillary Gel Electrophoresis (CGE) Methods ........................................... 24
       4.2.1 Polyacrylamide .................................................................................... 30
       4.2.2 Agarose ............................................................................................... 33
       4.2.3 Alkylcellulose and Other Polymers .................................................. 34
       4.2.4 Intercalators as Buffer Additives ....................................................... 37
       4.2.5 Ferguson Plots ..................................................................................... 39
       4.2.6 Instrument Parameters ....................................................................... 40
       4.2.7 Sample Injection and Matrix Effects; Quantitation ......................... 40
           4.2.7.1 Replaceable Gels ...................................................................... 40
           4.2.7.2 Non-Replaceable Gels ............................................................ 44
       4.2.8 Hybridization; Southern Blotting, Mobility Shift Assays .................. 46
4.3 Detection in CE: UV Absorbance vs. Laser-Induced Fluorescence (LIF) ................................................................. 49
  4.3.1 DNA Detection with LIF ................................................ 52
    4.3.1.1 Native and Indirect Fluorescence .......................... 52
    4.3.1.2 Intercalators ..................................................... 52
    4.3.1.3 Fluorescent Labeling ........................................... 55
  4.4 Fraction Collection: CE as a Micropreparative Tool .......... 57
    4.4.1 Fraction Collection Using Field Programming .......... 57
  5 Selected Applications .......................................................... 59
    5.1 Nucleotides, Nucleosides and Bases with CZE or MECC .... 59
      5.1.1 DNA Adducts; DNA Damage ................................. 59
      5.1.2 Nucleoside Analog Drugs ...................................... 59
      5.1.3 Nucleotides in Cell Extracts .................................. 61
      5.1.4 Increasing Detectability: LIF Detection .................. 63
    5.2 Purity Control of Synthetic Oligonucleotides ............... 63
      5.2.1 Phosphodiester Oligonucleotides .......................... 63
      5.2.2 Antisense DNA ................................................... 64
    5.3 DNA Sequencing ............................................................. 66
    5.4 dsDNA, PCR Products Analysis (< 2000 bp) ...................... 69
      5.4.1 Quantitation of Viral Load in Infectious Diseases ........ 70
      5.4.2 Competitive RNA-PCR by CE-LIF for Quantitation of Cellular mRNA ......................................................... 73
      5.4.3 Detection of DNA Polymorphisms and Mutations in Genetic Diseases ......................................................... 75
      5.4.3.1 Point Mutations .............................................. 78
      5.4.4 DNA Profiling in Forensic Work ............................... 80
      5.4.5 DNA Profiling of Plants, Bacteria and Fungi ............. 82
      5.4.6 Plasmid Mapping ................................................... 84
    5.5 dsDNA (2 to 20 kbp) by CGE .......................................... 85
      5.5.1 Quantitation of Plasmid Copy Number ...................... 86
    5.6 Very Large Chromosomal DNA (> 20 kbp) ....................... 86
  6 References ............................................................................... 87
About the Authors

Herbert E. Schwartz is a native of the Netherlands and received a Ph.D. in Analytical Chemistry from Northeastern University, Boston (under the direction of Prof. B. L. Karger), and a M.S. degree from the Free University, Amsterdam. He has been working in the analytical instrumentation industry for over 10 years, has authored approximately 50 publications in the field of separation science, and taught training courses in capillary electrophoresis. Before starting his consulting business, Palomar Analytical Services in Redwood City, California (tel: 415-365-3711; e-mail: heschwartz@aol.com) , Dr. Schwartz managed an applications group at Beckman Instruments, Inc., Palo Alto, California, and was involved in the development and marketing of the first fully automated, commercial capillary electrophoresis instrument at Microphoretic Systems. Prior to that, he was employed as a research chemist at Applied Biosystems and Brownlee Labs. He edited the previous primers (Volumes I–V) on capillary electrophoresis for Beckman.

Andras Guttman is a native of Hungary and a Principal Research Chemist with Beckman Instruments, Inc., Fullerton, California. His research currently focuses on the development of new chemistry kits for capillary electrophoresis, and size separations of protein and DNA molecules with gel-filled capillary columns. He received a Ph.D. in Analytical Biochemistry from the Hungarian Academy of Sciences, a M.S. degree from the University of Chemical Engineering at Veszprem, Hungary, and did post-doctoral work in Prof. B. L. Karger’s laboratory at the Barnett Institute, Northeastern University, Boston. Andras has authored 51 publications and holds 4 patents, mainly in the field of capillary electrophoresis. He joined the R&D group of the Biotechnology Development Center of Beckman Instruments, Inc., in 1990. During 1992–1993, he spent half a year in the Analytical Research Laboratory of Hafslund Nycomed Pharma in Linz, Austria, developing chiral separations by capillary electrophoresis.

Acknowledgments

We would like to thank Kathi J. Ulfelder at Beckman Instruments, Inc., Fullerton, California, for many suggestions and contributions to this book. The support of Jim McCoy and Dr. Nelson Cooke at Beckman is also highly appreciated. We thank Judy Strauss, LAHS, Los Altos, California, for reviewing drafts of the manuscript and Gale Leach and Annette Hurst at WordsWorth (Pacifica, California) for the desktop publishing of the manuscript.
Front Cover

The front cover shows EcoRI endonuclease, bound to a single-strand piece of DNA containing its recognition site sequence. The protein (1rle-pdb) is shown as a blue ribbon highlighting the secondary-structure regions; the space-filling representation of the DNA backbone is in yellow, and the nucleic bases in red. Those side-chains that interact with the nucleic acid are shown in thick-bond representation, in their element colors, i.e., carbon: green, oxygen: red, and nitrogen: blue. Courtesy of Dr. Don Gregory, Molecular Simulations, Burlington, Massachusetts.

Other Beckman primers (Volumes I, II, III, IV, V, and VI) on capillary electrophoresis:

<table>
<thead>
<tr>
<th>Title</th>
<th>Beckman Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction to Capillary Electrophoresis</td>
<td>360643</td>
</tr>
<tr>
<td>II. Introduction to Capillary Electrophoresis of Proteins and Peptides</td>
<td>266923</td>
</tr>
<tr>
<td>III. Micellar Electrokinetic Chromatography</td>
<td>266924</td>
</tr>
<tr>
<td>IV. Introduction to the Theory and Applications of Chiral Capillary Electrophoresis</td>
<td>726388</td>
</tr>
<tr>
<td>V. Separation of Proteins and Peptides by Capillary Electrophoresis: Application to Analytical Biotechnology</td>
<td>727484</td>
</tr>
<tr>
<td>VI. Introduction to Quantitative Applications of Capillary Electrophoresis in Pharmaceutical Analysis</td>
<td>538703</td>
</tr>
</tbody>
</table>

All trademarks and registered trademarks are the property of their respective owners.
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ac</td>
<td>alternating current</td>
</tr>
<tr>
<td>ACE</td>
<td>affinity capillary electrophoresis</td>
</tr>
<tr>
<td>ARMS</td>
<td>amplification refractory mutation system</td>
</tr>
<tr>
<td>ASA</td>
<td>allele-specific amplification</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CGE</td>
<td>capillary gel electrophoresis</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyltriethylammonium bromide</td>
</tr>
<tr>
<td>CZE</td>
<td>capillary zone electrophoresis</td>
</tr>
<tr>
<td>dc</td>
<td>direct current</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>EB</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EOF</td>
<td>electroosmotic flow</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GE</td>
<td>gel electrophoresis</td>
</tr>
<tr>
<td>HPA</td>
<td>heteroduplex polymorphism analysis</td>
</tr>
<tr>
<td>HPCE</td>
<td>high-performance capillary electrophoresis</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HPMC</td>
<td>hydroxypropylmethylcellulose</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>ITP</td>
<td>isotachophoresis</td>
</tr>
<tr>
<td>kbp</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolts</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LIF</td>
<td>laser-induced fluorescence</td>
</tr>
<tr>
<td>MCAD</td>
<td>medium-chain coenzyme A dehydrogenase</td>
</tr>
<tr>
<td>MECC</td>
<td>micellar electrokinetic capillary chromatography</td>
</tr>
<tr>
<td>PA</td>
<td>polyacrylamide</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PNA</td>
<td>peptide nucleic acid</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription / reverse transcribed</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>SSCP</td>
<td>single-stranded conformation polymorphism</td>
</tr>
<tr>
<td>STR</td>
<td>short tandem repeat</td>
</tr>
<tr>
<td>TTAB</td>
<td>tetradecyltrimethylammonium bromide</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable number tandem repeat</td>
</tr>
</tbody>
</table>

PCR is covered by U.S. patents owned by Hoffmann-La Roche, Inc.
1 Introduction

Electrophoresis is the transport process of charged analytes under the influence of an electric field. Nucleic acids are generally electrophoresed in neutral or basic buffers—as anions with their negatively charged phosphate groups (at acidic pH, polynucleotides are insoluble in water). Historically, electrophoresis experiments in glass tubes were reported as early as the beginning of the 19th century. Before the advent of rod and slab gels, early 20th century electrophoresis was performed in free solution. In the mid 1930s, the Swedish chemist Arne Tiselius applied a free solution technique—moving boundary electrophoresis—to serum protein analysis, work for which he would later receive the Nobel Prize (Tiselius, 1937). Studies with “Tiselius”-type apparatus equipped with Schlieren optics to visualize nucleic acid separations continued during the 1940s and 1950s (see, for example, Chargaff and Saidel, 1949). Ever since the publication of DNA’s double helical structure (Watson and Crick, 1953), electrophoresis has been a standard, indispensable analytical tool in modern biochemistry and molecular biology; electrophoretic procedures are used in almost every aspect of basic or applied biomedical and clinical research.

Since the 1960s, methods employing supporting media, such as polyacrylamide or agarose gels, have become the norm for protein and nucleic acid analysis. The gel matrix acts as an anticonvective medium, reducing convective transport and diffusion so that separated sample components remain positioned in sharp zones during the run. In addition, the gel acts as a molecular sieve, separating nucleic acids according to their size. Electrophoresis, as performed today in the majority of laboratories, is still typically a manual process: the gels are poured, the separation is run, and the bands are visualized by means of a staining/destaining process; a photograph of the gel is then kept for records. This makes electrophoretic procedures often time consuming and labor intensive. With the exception of DNA sequencing, electrophoretic procedures typically have not been fully automated, unlike chromatographic separation methods such as HPLC. In addition, most electrophoretic techniques are qualitative and accurate quantitation is often problematic.

Capillary electrophoresis (CE) is a relatively new, powerful separation technique that is ideally suited for handling small amounts of sample material. This demand becomes increasingly prominent in bioanalytical research, e.g., in biotechnology and in various clinical, diagnostic, genetic,
and forensic applications. In its present instrumental configuration, CE is more than a decade old (Jorgenson and Lukacs, 1981, 1983). Preceding modern CE, it is interesting to note that, as early as 1953, some experimenters recognized the importance of miniaturized equipment when dealing with certain biological samples. For example, ribonucleotides could be identified in single cells using “micro-electrophoresis” in fine silk fibers (Edstrom, 1953). Classical electrophoretic techniques were not suitable because the sample was too small in size.

CE offers several similarities to high performance liquid chromatography (HPLC), i.e., ease of use, high resolution, speed, on-line detection, and full automation capability. CE, having taken essential components from both HPLC and electrophoresis, can be viewed as an instrumental approach to electrophoresis. In its relatively short history, CE has found particular applicability in bioanalysis. Analogous to HPLC, CE is also often referred to as high performance capillary electrophoresis (HPCE). A number of recent text books cover the history, theory, instrumentation, and applications of CE (Camillieri, 1993; Guzman, 1993; Landers, 1994). A new journal—*J. Cap. Elec.*—(Guzman, 1994) has been recently introduced to cover novel applications in the field. Other Beckman primers on CE are listed at the start of this book.

While the first papers on DNA analysis by CE only appeared in 1988 (Kasper *et al*., 1988; Cohen *et al*., 1988), their number since then has grown exponentially. Partly, this has been fueled by the emergence of new tools in molecular biology, in particular PCR, and the Human Genome Project, with its spin-offs such as gene hunting technology. It is rapidly becoming clear that many, if not all, slab gel electrophoresis techniques can be transferred to a capillary format. Benefits of this transformation would include fast, high-resolution analyses, full automation and data storage capability; in addition, only minute amounts of sample are required with CE techniques. With regard to the latter, the recent introduction of a highly sensitive, laser-induced fluorescence (LIF) detector has opened up new perspectives for low-level DNA analysis, *e.g.*, detectability into the zeptomole (10⁻²¹ mol) range.

This book describes the main principles of the various CE methods and their applications to nucleic acid analysis, specifically those dealing with nucleosides, nucleotides, ss oligonucleotides, and dsDNA (PCR) fragments. Whereas the emphasis is on DNA, some RNA separations are also discussed, as well as applications where RNA is reverse-transcribed
into DNA. In addition, a brief overview of other high-resolution, complementary techniques (HPLC, slab gel electrophoresis) will be given. The main focus is on applications which can be performed with today’s commercially available instrumentation (as opposed to those with special, homemade designs). Instrumentation will not be discussed extensively; additional information on CE can be found in the above-mentioned textbooks.
2 Fundamentals of Capillary Electrophoresis (CE)

2.1 CE Instrumentation

Figure 1 shows a schematic diagram of a CE system such as that used in the P/ACE™ Capillary Electrophoresis Systems from Beckman. Fused silica capillaries, generally 20–100 μm i.d. and 20–100 cm long, are used as the separation channel. The inner surface of the capillaries can be untreated or coated, depending on the application, and are encased in a temperature-controlled (± 0.1°C) cartridge. The capillary inlets and outlets are positioned in the sample and/or buffer vials. In P/ACE, sample introduction (often called “injection” to maintain the analogy with HPLC) is either by pressure or by electrokinetic means; the samples are loaded in an autosampler tray. In the pressure method, a sample vial is temporarily pressurized to allow the flow of sample into the capillary; the electrokinetic method utilizes the electric field to draw charged analytes into the capillary.

![Diagram of a CE instrument with normal polarity (anode at injection side).]
In contrast to slab gel electrophoresis, CE can take advantage of two types of driving forces: 1) the force causing the electrophoretic migration; and 2) the force exerted by the electroosmotic flow (EOF) through the capillary. The EOF bulk flow results from the charged inner capillary wall during application of an electric field (Figure 2). With untreated, fused-silica tubing and an aqueous buffer, a negatively charged capillary surface is obtained. The magnitude of the EOF is dependent on various experimental factors, most notably the pH of the buffer solution. Capillary coatings can reverse, reduce, or even totally eliminate the EOF. The latter is the case in gels (anticonvective medium) when DNA restriction fragments, synthetic oligonucleotides, or DNA sequencing fragments are separated by capillary gel electrophoresis (CGE).

![Figure 2. Schematic of electrophoresis and electroosmosis in a separation of anionic, neutral, and cationic analytes.](image)

Most modern CE instruments allow voltages of up to 30 kV during the runs. CE voltages are generally much higher than those used in slab gel electrophoresis. This accounts, in part, for the high resolution and fast analysis times of CE separations. Joule heat resulting in the capillaries is effectively dissipated through the capillary wall by the surrounding cooling liquid (as in P/ACE). Detection is achieved by monitoring UV absorbance directly on-capillary through a window in the capillary. Other commercially available detection options include laser-induced fluorescence (LIF), UV-Vis scanning diode array, and mass spectrometry.
The capillary cartridge format used in P/ACE allows for controlling the temperature (within 0.1°C) of the capillary column, which is very important for obtaining good run-to-run reproducibility (Nelson et al., 1989). Usually, CE separations are performed at 20 to 30°C, i.e., room temperature, although P/ACE permits temperature settings between ≈ 15 to 50°C for special applications. Other features of the P/ACE system are the possibility of fraction collection and of field programming. While it is primarily used for analytical separations, CE can also be used for micropreparative DNA work (see Section 4.4). Similarly to HPLC, on-line system control and computerized data processing are incorporated.

2.2 CE Modes

Different separation modes, schematically shown in Figure 3, have been developed for CE. For each of these CE modes, Table 1 shows the main principle of operation and the applicability for DNA analysis. Isoelectric focusing (IEF) will not be discussed as this technique is almost exclusively used for peptides and proteins. CZE (capillary zone electrophoresis), MECC (micellar electrokinetic capillary chromatography), and ITP (isotachophoresis) are free-solution techniques. With CZE, only charged species can be separated. MECC is particularly useful for the separation of relatively small, neutral molecules such as most pharmaceuticals. Bases, nucleosides, nucleotides, and small oligonucleotides (< 10 bases) also have been separated by MECC, as will be discussed in Section 5.1. The technique makes use of surfactants (e.g., sodium dodecyl sulfate [SDS] or cetyltrimethylammonium bromide [CTAB]) which, when added to the run buffer in sufficiently high enough concentrations, form micelles in solution. During the high-voltage run, the micelles migrate in the opposite direction to the EOF which drives the separation (Figure 4). Analytes partition into the micelles differently according to their hydrophobicity, thus yielding different elution times. For small DNA fragments, e.g., nucleosides, nucleotides, and small oligonucleotides, free-solution techniques (CZE, MECC) can be applied—generally in conjunction with uncoated capillaries.
Figure 3. Modes of CE. BGE = background electrolyte; LE = leading electrolyte; TE = terminating electrolyte; A, B = sample components. t = 0, start of separation (injection); t = x, separation after time x.

Figure 4. Schematic of the separations principle of MECC. The detector window is assumed to be positioned near the negative electrode.
Isotachophoresis (ITP) or displacement electrophoresis was developed in capillaries in the 1960s and 1970s (Everaerts, 1976). The sample is placed between carefully selected leading and terminating electrolytes (Figure 3); during migration, the sample concentrations are adapted to that of the leading zone. ITP can be viewed as a front runner to modern CE—many of its principles apply to modern CE techniques. Except for its utility as a preconcentration technique with CZE (e.g., in nucleotide separations—see Sections 4.1.2 and 5.1), ITP will not be discussed in this book. For larger DNA (e.g., antisense DNA, sequencing fragments, restriction fragments), sieving techniques with gels (“polymer networks”) are required. These CGE applications, requiring special pretreated (coated) capillaries, are discussed later in Sections 4.2 and 5.
2.3 Theory

2.3.1 Migration Velocity

When a uniform electric field \( E \) is applied to a polyion with a net charge of \( Q \), the electrical force \( (F_e) \) is given by

\[
F_e = QE
\]  

(1)

In free solution, as well as in viscous media such as a crosslinked gel or a non-crosslinked polymer solution, when the ion is set in motion by the applied electric field, a frictional force \( (F_f) \) acts in the opposite direction:

\[
F_f = f(dx/dt)
\]  

(2)

where \( f \) is the translational friction coefficient and \( dx \) and \( dt \) are the distance and time increments, respectively.

Differences in sample properties such as shape, size, or net charge result in different electrophoretic mobilities and provide the basis of electrophoretic separation. The migration of the charged solute under the electric field is expressed according to Newton’s second law as:

\[
m(d^2x/dt^2) = EQ - f(dx/dt)
\]  

(3)

i.e., the product of the mass \( (m) \) and acceleration \( (d^2x/dt^2) \) is equal to the difference of the electrical and frictional forces. When the force from the applied electric field on the charged solute is counterbalanced by the frictional force, the solute migrates with a steady state velocity \( (v) \):

\[
v = dx/dt = EQ/f
\]  

(4)

The translational friction coefficient is influenced by the temperature-dependent \( (T) \) viscosity of the separation matrix, especially in free solution and in non-crosslinked polymer networks:

\[
f = C_1 e^{(-1/T)}
\]  

(5)

where \( C_1 \) is constant for a given shape solute (Guttman et al., 1994). In capillary gel electrophoresis, the size-dependent retardation of the solute is a primary function of the separation polymer concentration \( (P\%) \):

\[
v = v_0 e^{(-K_R P\%)}
\]  

(6)

where \( v_0 \) is the free solution velocity of the solute and \( K_R \) is the retardation coefficient (Chrambach, 1985; Andrews, 1986).
As a first approximation, the total electrostatic force \( F_e \) on the DNA molecule can be assumed to be constant per unit length, since each residue has the same net negative charge. Thus, \( Q \) is proportional to the number of bases \( (n) \) in the molecule (Cantor et al., 1988) and, therefore, one can consider using the number of bases in the DNA molecule \( (n) \) instead of the net charge:

\[
F_e = EQ \approx En^k
\]  

(7)

where \( k \), the exponent of \( n \), represents information about the apparent shape of the DNA molecule under the electric field used, \textit{i.e.}, random coil, oriented, stretched, etc. (Lerman and Frish, 1982, Slater and Noolandi, 1988, Stellwagen, 1989).

Combining Equations 4–7, and incorporating all the constants together in one \( (\text{Const}) \), the electrophoretic velocity of a migrating DNA molecule can be described as:

\[
v = \text{Const} \, En^k \, e^{-1/T} \, e^{(-KRP)}
\]  

(8)

where \( En^k \, e^{-1/T} \) corresponds to the free solution velocity \( (v_0) \) at zero polymer concentration and \( e^{(-KRP)} \) represents the molecular sieving effect.

If there is any special additive in the gel, such as a complexing agent, the solute will have a distribution between the complex and the electrolyte:

\[
v = \text{Const} \, En^k \, e^{-1/T} \, e^{(-KRP)} \, R_p \, (1 + K_L)^{-1}
\]  

(9)

where \( K_L \) is the complex formation constant of the L ion and \( R_p \) is the molar ratio of the free solute (Guttmann and Cooke, 1991 A). Depending on the charge and size of the complexing agent, the complex may migrate faster or slower than the free solute. When the complexing agent has a charge opposite to that of the solute, then an uncharged complex may be formed—therefore, the higher the concentration of the complexing agent, the slower will be the migration velocity of the solute.

In capillary gel electrophoresis, molecular sieving can be described by the Ogston theory (Ogston, 1958): the average pore size of the matrix is in the same range as that of the hydrodynamic radius of the migrating solute. In this case the logarithm of the velocity of the migrating solute is proportional to the size of the solute:

\[
v \approx E \, e^{-n}
\]  

(10)

This theory also assumes that the migrating particles behave as unperturbed spherical objects.
On the other hand, it is well documented that large biopolymer molecules with flexible chains, e.g., DNA, can still migrate through a polymer network which has a pore size that is significantly smaller than the size of the solute. This phenomenon can be explained by the reptation model. The reptation model describes the migration of the polyion, suggesting a “head-first” motion through the pores of the polymer network (Lerman and Frish, 1982; DeGennes, 1979; Lumpkin et al., 1985; Viovy and Duke, 1993).

According to the reptation model, the velocity of the migrating solute is inversely proportional to the size of the solute, i.e., the chain length:

\[ v \approx \frac{E}{n} \quad (11) \]

When higher electric field strengths are used, “biased” reptation occurs, in which case the velocity of the solute can be described as:

\[ v \approx \frac{E}{n} + bE^2 \quad (12) \]

where \( b \) is a function of the mesh size of the polymer network as well as the charge and segment length (Grossman et al., 1992) of the migrating DNA molecule.

### 2.3.2 Efficiency and Resolution

According to theory, the major contributor to band broadening, besides the injection and detection extra-column broadening effects, is the longitudinal diffusion of the solute in the capillary tube (Jorgenson and Lukacs, 1983; Terabe et al., 1989). The theoretical plate number \( N \) achieved is characteristic of the column efficiency:

\[ N = \frac{\mu_e E l}{2D} \quad (13) \]

where \( \mu_e \) is the electrophoretic mobility, \( D \) is the diffusion coefficient of the solute in the separation buffer system, and \( l \) is the effective column length.

Resolution \( (R_s) \) between two peaks can be calculated from the differences of their electrophoretic mobility \( (\Delta \mu_e) \) (Karger et al., 1989):

\[ R_s = 0.18 \ \Delta \mu_e \left( \frac{E l}{D \mu_{em}} \right)^{1/2} \quad (14) \]

where \( \mu_{em} \) is the mean mobility of those species.

Equations 13 and 14 demonstrate that higher applied electric field and lower solute diffusion coefficient would result in higher efficiency \( (N) \) and higher resolution \( (R_s) \). The limiting factor in efficiency is mainly the heat
generated \( Q_j \), Joule heating) from the power applied \( P = V \times I \) to the narrow bore capillary tubing (Nelson et al., 1989):

\[
Q_j = \frac{P}{r^2IL}
\]  

(15)

where \( I \) is the current, \( L \) is the total column length and \( r \) is the column radius.

Because of the sensitivity of the electrophoretic mobility to temperature \((\approx 2\% \text{ per } ^\circ\text{C})\), as well as the temperature dependence of the complex formation constant, good temperature control is extremely important for achieving good migration reproducibility (Nelson et al., 1989; Karger et al., 1989).
3 Separation of DNA by Techniques Other Than CE

Separation methods of biomolecules can be divided into three major categories: 1) methods which take advantage of size differences of the analytes (e.g., gel filtration, ultrafiltration, centrifugation); 2) methods based on electrical charge differences (e.g., ion exchange chromatography); and 3) methods based on differences in the specific biochemical properties of analytes (e.g., hybridization, immunoadsorption techniques).

In electrophoresis, chromatography, and centrifugation the separation mechanism is based on differences in transport velocities of the analytes. Theoretically, therefore, many analogies exist between these separation techniques. The driving force to achieve the analyte transport is an electric field in electrophoresis, a hydrodynamic force (flow) in chromatography, and a centrifugal force in centrifugation. Frictional resistance determines the extent of retardation during the transport in electrophoresis or centrifugation, while adsorption plays a major role in chromatography. Low-resolution techniques, e.g., centrifugation, are primarily used for purification of DNA. Membrane technologies are frequently used to remove unwanted components from the sample (e.g., desalting by ultrafiltration) or to transfer DNA bands from gels for further manipulation (e.g., in blotting). A comprehensive discussion of all separation techniques for DNA is beyond the scope of this book. In the following sections, the high-resolution separation techniques (i.e., HPLC and slab gel electrophoresis) are briefly reviewed, and some comparisons and analogies with CE are made.

3.1 HPLC

In liquid chromatography, the separation mechanism involves partitioning of analytes between a mobile and stationary phase. The selectivity of the separation is governed by the partitioning of the analytes in the stationary phase. Diffusion characteristics of the analyte play an important role: generally the higher the molecular mass of the analyte, the lower the separation efficiency (as expressed by the number of theoretical plates). It can be theoretically shown that CE—and electrophoresis in general—should have distinct advantages over HPLC (Jorgenson and Lukacs, 1983) when large biomolecules (proteins, DNA, polysaccharides) are separated. This is mainly due to the relatively slow diffusion characteristics of large molecules which work in CE’s favor. The mass transport of large biomolecules
in the HPLC mobile phase is slow (relative to the speed of mass transfer), resulting in increased band broadening. In gel electrophoresis, the problems related to mass transfer do not exist. Separation efficiencies in CGE—when expressed as numbers of theoretical plates, \( N \)—can be extremely high (e.g., \( N \approx 30 \) million per meter for oligonucleotides, Guttman et al., 1990). With very large DNA fragments (> 10,000 bp), resolution and efficiency decrease, as predicted (Bae and Soane, 1993).

It is important to note, however, that selectivity often is more important than “raw” separation power. In electrophoresis, therefore, it is important to find ways to optimize the difference in electrophoretic mobilities (e.g., with MECC), control the EOF, use certain capillary coatings, etc. Generally, in HPLC, the elution of large molecules can only be achieved by changing the selectivity during the separation process, i.e., by means of gradient elution. Instrumentation in HPLC is rather complex compared to the simplicity of a CE system. The separation channel in CE is an open or gel-filled tube; in HPLC, expensive prepacked columns are required which have a limited lifetime. Nevertheless, impressive separations of DNA are possible with HPLC. Size-dependent chromatographic separations of nucleic acids have recently been reviewed by Kasai (1993). At present, the most popular types of HPLC for DNA separations are anion-exchange chromatography with 2.5 µm, nonporous, polymeric particles (for DNA < 25 kbp), hydrophobic interaction chromatography (for DNA < 3 kbp), and gel permeation chromatography (for DNA < 6 kbp). A recently developed new mode, “slalom chromatography,” appears promising for DNA in the 5 to 50 kbp range. Other papers by Baba and co-workers (1991), Oefner and Bonn (1994), and Katz (1993) also discuss the state of the art in HPLC of DNA, while comparing HPLC with slab gel electrophoresis or CE.

As mentioned in the Introduction (Section 1), HPLC procedures are often complementary to those developed by electrophoresis because the separation mechanisms are different. Both methodologies can be fully automated. With the Gold™ Software package from Beckman, HPLC and CE autosamplers can be operated simultaneously from one computer. In general, HPLC procedures are easier to scale up to (semi)preparative work, although CE also has been used for purifying nanogram to microgram amounts of DNA material (Section 4.4). HPLC of PCR products has been presented as being more quantitative than slab gel electrophoresis-densitometry (Zeillinger et al., 1993). It also has been claimed that HPLC is a more quantitative technique than CE. However, in our opinion, this has more to do with CE maturing as an analytical technique: when operational
parameters are fully understood and controlled, precision, accuracy, and quantitation are as good as those obtained with HPLC (see also Sections 5.4.1 and 5.4.2). Detection sensitivity is dependent on the type of detector used, as well as the volume amount loaded onto the column: with UV detection, minimum detectable concentrations of 300 ng/mL (for a 404 bp DNA fragment, 20 µL sample loop) have been reported, similar to detectability feasible with CE-UV detection. HPLC with fluorescence detection, combined with fluorescent labeling, is significantly more sensitive, and could be applied to low-level DNA detection (Oefner and Bonn, 1994).

3.2 Gel Electrophoresis: Polyacrylamide (PA) and Agarose

3.2.1 Instrumentation and Detection

From all variants of electrophoresis, slab gel techniques have proven one of the most popular for DNA. In the 1970s, polyacrylamide and agarose became the media of choice for separating proteins and nucleic acids. Horizontal as well as vertical formats of slab gels are used, as well as cylindrical rods for certain applications (Rickwood and Hames, 1983; Andrews, 1986). After electrophoresis of the DNA (the process is stopped after the marker reaches the edge of the gel), the gel is typically soaked in a solution of ethidium bromide (or other fluorescent dye), washed to remove unbound dye, illuminated with UV light, and photographed to reveal the fluorescence of dye-bound DNA. (Note that in CE the separation and detection of DNA takes place simultaneously, i.e., in real time. Some slab-gel-based instrumentation also allows real-time detection of DNA, e.g., in automated DNA sequencing).

Another sensitive, albeit time-consuming, detection technique, autoradiography, is often used to identify specific DNA sequences, e.g., those in disease-causing genes or DNA sampled from crime scenes. The procedure, known after its inventor as “Southern blotting” (Southern, 1975), involves the use of radiolabeled DNA probes which target complementary DNA strands present in the original sample. One forensic typing methodology uses Southern blotting with autoradiography for identification (“DNA fingerprinting”) of materials found at crime scenes. This method, known as restriction fragment length polymorphism (RFLP) typing, is shown in Figure 5.
Figure 5. DNA profiling process using multistrip restriction fragment length polymorphism (RFLP) analysis. Source: Lifecodes Corporation, Stamford, CT.
Slab gels have the distinct advantage of running multiple samples simultaneously. This, of course, increases sample throughput compared to instrument designs where the sample has to be assayed one at a time, such as HPLC or CE. However, CE has—because of the higher fields applied—much faster run times than slab gel electrophoresis (≈ 10 to 100 times faster). Instrumentation allowing samples to be run simultaneously on multiple CE columns has also been described recently and applied to PCR fragments and DNA sequencing (see also Section 5.3). Beckman Instruments, Inc., has already previewed a CE instrument with seven capillaries designed for clinical applications.

3.2.2 Separation Mechanism

While the original purpose of the gel was to provide an anticonvective medium for the electrophoresis, the gels resolve DNA fragments (and also SDS-complexed proteins) by acting as molecular sieves. The amount of sieving can be controlled by adjusting the concentration of the gel. With polynucleotides, the phosphate group of each nucleotide unit carries a strong negative charge that is much stronger than any of the charges on the bases above pH 7. The mass-to-charge ratio of the polynucleotides is independent of the base composition and, consequently, nearly the same for closely related species. For that reason, free-solution techniques (i.e., the electrophoretic medium contains no gel or polymer network solution) have not proven successful in the electrophoresis of oligonucleotides or dsDNA.

The actual mechanism involved in DNA separations in electrophoresis has been—and is still—the topic of much discussion (see, for example, Stellwagen, 1987; Slater and Noolandi, 1989; Smisek and Hoagland, 1990; Barron et al., 1994). Fluorescent video microscopy allows individual DNA molecules to be monitored as they sieve through a gel under the influence of an electric field (Smith et al., 1989). However, no single model can fully account for the dependence of DNA mobility on its molecular size and a number of experimental parameters (field strength, gel concentration, etc.). Two models have been proposed which are shown schematically in Figure 6 (see also Theory, Section 2.3.1, Equation 10). The first, commonly referred to as the “Ogston” mechanism, involves a coil of DNA percolating through a network of polymer fibers. It is thought that the coil moves through the gel as if it were a rigid, spherical particle. Its electrophoretic mobility is proportional to the volume fraction of the pores of a gel that the DNA can enter. Since the average pore size decreases with increasing gel concentration, mobility decreases with increasing gel concentration and increasing molecular weight. The Ogston theory does not account for the fact that a relatively large DNA molecule may stretch or deform so it can squeeze its way through the pores.
Figure 6. (A) Schematic diagram of a solute migrating through a polymer network by the Ogston mechanism. The DNA percolates through the mesh as if it were a rigid particle. (B) Schematic diagram of a solute migrating by the reptation mechanism. In this case, the DNA is forced to squeeze through the tubes formed by the polymer network. Reproduced with permission from Grossman, P. D., and Soane, D. S., Biopolymers 31, 1221 (1991).

In the reptation model, the DNA is thought to squeeze through the mesh of the gel as if it were a snake going through an obstacle course (see Equation 11 in Section 2.3.1). The “biased” reptation model (Slater and Noolandi, 1989) can account for the experimentally observed fact that the mobility of DNA becomes independent of the molecular weight at high field strength (Figure 7). At high field strengths, the DNA stretches and the dependence of mobility on molecular size decreases (see Equation 12 in Section 2.3.1). However, the reptation theory cannot explain certain effects observed in pulsed-field electrophoresis (Smisek and Hoagland, 1990).

Figure 7. Schematic illustration showing the elongation of DNA under the influence of the electric field (reptation). Reproduced with permission from Grossman, P. D., and Soane, D. S., Biopolymers 31, 1221 (1991).
Different gel matrices have been used for separating DNA. The two most common ones are based on either polyacrylamide or agarose and they will be discussed next. Interestingly, gels are currently not the only medium for electrophoresis of DNA: Volkmuth and Austin (1992) recently demonstrated—using a microlithography technique—that DNA can electrophorese in arrays of micron-sized posts on silicon chips. This “obstacle course” is a highly regular and well-characterized medium for DNA study.

### 3.2.3 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels are most often used for relatively small DNA fragments (DNA sequencing gels, restriction fragments), small RNA molecules and proteins (SDS-PAGE). Polyacrylamide is usually prepared by crosslinking acrylamide with N,N'-methylenebisacrylamide in the cast with the shape of a slab or a rod in which the electrophoresis is to be carried out (“pouring of the gel”). Linear polyacrylamide (i.e., non-crosslinked) has also been used with classical slab gel or column techniques (Bode, 1977; Tietz et al., 1986), but has not found widespread utility. However, it has been found to be extremely useful when used in capillaries, i.e., in modern CE, where it is used as a replaceable, relatively non-viscous, polymer network in capillaries (see Section 4.2).

### 3.2.4 Agarose Gel Electrophoresis

Agarose has larger pores than PA and is generally used for the separation of relatively large DNA molecules. It is a natural product (a polysaccharide) made from agar-agar which is isolated from algae. Agarose gels tend to have high mechanical strength and biological inertness. Unlike, PA, agarose does not become crosslinked during the gelation process. A 0.8% agarose gel is sufficiently rigid that it can separate DNA fragments with MWs of up to 50 million, while dilute 0.2% agarose gels have been applied to fragments of up to 150 million MW. Although agarose has been used with CE (Bocek and Chrambach, 1991, 1992), it has—in contrast to PA—not yet been widely applied; prepacked capillaries or gel kits with agarose are not yet commercially available.

### 3.2.5 Pulsed-Field Gel Electrophoresis (PFGE)

Gel electrophoresis with dc fields can separate DNA fragments up to ≈ 30 kb. Larger DNA fragments (up to ≈ 6 Mb in size, chromosomal DNA) can be resolved by using ac fields, i.e., in pulsed-field gel electrophoresis (PFGE; see Gardiner, 1991, for a review). The principle of PFGE is
sketched in Figure 8. It is interesting to mention that the MW range of electrophoresis (as extended by PFGE) is much greater than that possible with any mode of chromatography; here the liquid flow that drives the separation causes shearing of the fragile, large DNA. The problems encountered with “normal” electrophoresis when large DNA is separated have been attributed to the stretching and reptation schematically shown in Figure 7. Under these conditions, the molecular sieving mechanism is no longer operative. However, by pulsing the electric field, the reptation of DNA is counteracted. At the time of this writing, research in CE with pulsed fields has just begun (Sudor and Novotny, 1994; Kim and Morris, 1994). Pulsed-field CE separations with λDNA standards (8.3–48.5 kb) and restriction-digested λDNA (48.5 kb to 1 Mb) were \( \approx \) 10 to 50 times faster than typical slab gel separations of this kind.

\[
\begin{array}{c}
\text{A} - \\
\downarrow \\
\text{B +} \\
\downarrow \\
\text{A +} \\
\end{array}
\]

\[
\begin{array}{c}
\text{B} + \\
\downarrow \\
\text{A -} \\
\downarrow \\
\text{B -} \\
\end{array}
\]

Figure 8. Diagram of pulsed-field electrophoresis of DNA in an agarose gel. The dotted lines indicate the sample wells. A and B represent two sets of electrodes. When the A electrodes are on, the DNA is driven downward and to the right. When the A electrodes are turned off, the B electrodes are activated, which causes the DNA to move downward and to the left. Reproduced with permission from Gardiner, K., Anal. Chem. 63, 658 (1991). Copyright: American Chemical Society.
4 CE Methods: Principles and Strategies

In this section, a brief overview will be given of the CE systems used in the various type of nucleic acid applications. Generally, untreated capillaries are used in conjunction with the free-solution techniques of CZE or MECC. They are applied to the separations of bases, nucleosides, or simple oligonucleotides. Larger oligonucleotides (> 10 mers), antisense DNA, and dsDNA (restriction fragments, PCR products, etc.) require gel-filled capillaries for molecular sieving.

4.1 Free Solution Methods

4.1.1 CZE with Untreated or Coated Capillaries

In most applications of CE involving small molecules (also peptides and proteins), untreated, fused-silica capillaries are used. When the capillary is filled with solution, a negative charge on the wall surface prevails (Figure 9A). In P/ACE, the detection window is positioned near the cathode (negative electrode) at \( \approx 7 \) cm from the capillary outlet. This configuration is referred to as the “normal polarity” mode. The electroosmotic flow (EOF) generated with untreated capillaries is strongly pH dependent, and varies according to an S-function (at high pH, EOF is relatively large); the direction of the EOF in an untreated capillary is always toward the cathode (detector side). When a neutral buffer (e.g., phosphate, pH 7) is used for nucleotide separations, the electrophoretic flow forces the negatively charged nucleotides toward the anode, opposite to the direction of the EOF (the zone of anions in Figure 2). Detection of the nucleotides will only take place if the existing EOF is larger than the electrophoretic flow \( (\mu_{\text{EOF}} > \mu_{e}) \).
Using a solution of cationic surfactants (e.g., CTAB, DTAB), the surface of the fused-silica capillary can be modified *in situ* from a negative to a positive surface charge (Figure 9B). The capillary wall is said to be “dynamically” modified. When the concentration of the surfactant in the buffer is below its critical micelle concentration (CMC), CZE conditions exist. When the CMC is exceeded, a pseudo-stationary, micellar phase is created in the capillary and MECC conditions prevail; the charge on the capillary—and therefore the direction of the EOF—is reversed, as shown in Figure 9B. To drive the EOF again toward the detector, the polarity of the power supply must be reversed (cathode at the injection side—“reversed polarity mode”).

Generally, the strategy with permanently coated capillaries is to moderate or entirely eliminate the EOF. The result is often a more efficient and reproducible separation. Polyacrylamide-coated capillaries have been used for nucleotide separations in the CZE mode (Huang *et al.*, 1992). The eCAP™ Neutral capillary (P/N 477441) from Beckman—which also has a polyacrylamide-based, hydrophilic coating—should also prove useful for these types of separations.
4.1.2 CZE with Sample Stacking or ITP Preconcentration

In classical electrophoresis, sample preconcentration (“enrichment”) can be achieved with discontinuous buffer systems (Ornstein, 1964; Andrews, 1986). Similar tricks to obtain an increased sample detectability can also be applied in CE (Chien and Burgi, 1989; Albin et al., 1993). This is especially useful when the analytes’ concentrations are very low (e.g., in biological media) and UV detection is pushed to its limits (note: LIF detection may be a good alternative in these cases as will be discussed in Section 4.3.1). Dissolving the sample components in a matrix which has a ≈ 10 X lower ionic strength than the run buffer may result in sharper peaks and 2 to 3 X better detectability (Figure 10). Other similar stacking effects can be obtained by manipulating the pH of the sample vs. that of the run buffer (Abersold and Morrison, 1990) or by using electrokinetic injection (Schwartz et al., 1991).

![Diagram of sample stacking mechanism](image)

**Figure 10.** The sample “stacking” mechanism. Sample ions have an enhanced electrophoretic mobility in a lower conductivity environment (i.e., elevated local field strength. When a voltage is applied, anions (e.g., polynucleotides) in the sample plug instantaneously accelerate toward the adjacent separation buffer zone where, on crossing the boundary, a higher-conductivity environment (lower field strength) causes a decrease in electrophoretic velocity and “stacking” of the sample ions into a buffer zone smaller than the original sample plug. Adapted with permission from Oda and Landers, Handbook of Capillary Electrophoresis, Landers (Ed.), Boca Raton, CRC Press, 1994.
More dramatic increases in sample detectability (≈ 100 times) can be achieved by applying the principles of isotachophoresis (ITP) to CE. ITP can be used as a preconcentration step prior to separation in the CZE mode. Using homemade systems, Foret and co-workers (1990) have shown the utility of ITP-CE for the separation of nucleotides. ITP-CE can be performed in commercial CE instruments such as P/ACE. The leading and terminating electrolytes must be carefully selected to achieve the desired zone-focusing effect (Schwer and Lottspeich, 1992; Foret et al., 1992, 1993).

4.1.3 Micellar Electrokinetic Capillary Chromatography (MECC)

MECC takes advantage of the differential partitioning of analytes into a pseudo-stationary phase consisting of micelles. The principle of this technique was discussed earlier (Figure 4). Anionic surfactants such as SDS are typically used in concentrations of 10 to 200 mM. When cationic surfactants such as CTAB are used, the EOF is reversed; therefore, the polarity of the power supply also must be reversed to attain the same flow direction from the inlet to the outlet. MECC conditions appear particularly useful in the analysis of nucleotides in biological media such as cell extracts (see Section 5.3.1).

4.2 Capillary Gel Electrophoresis (CGE) Methods

A gel is often somewhat vaguely defined: “a form of matter intermediate between a solid and a liquid” (Tanaka, 1981). Gels may vary in consistency from viscous fluids to fairly rigid solids. In the electrophoresis literature, the nomenclature is also rather ambiguous: terms such as “polymer solutions,” “polymer networks,” “entangled polymer solutions,” “chemical gels,” “physical gels,” and “liquid gels” all have been used to describe gel media. In addition, the term “replaceable” gel recently has been introduced to describe relatively non-viscous gels that can be rinsed in and out of the capillary. In CE, two types of gel matrices can be distinguished: 1) a relatively high-viscosity, crosslinked gel that is chemically anchored to the capillary wall (“chemical” gel), and 2) a relatively low-viscosity, polymer solution (“physical” gel). With both types of gels, precoated capillaries are used to eliminate the EOF. Table 2 summarizes the main differences between these gels. Figure 11 illustrates the formation of physical and chemical polymer networks.
### Table 2. Characteristics of Gel Matrices Used in CGE

<table>
<thead>
<tr>
<th><strong>Chemical Gels</strong></th>
<th><strong>Physical Gels</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Crosslinked and/or chemically linked to the capillary wall</td>
<td>• Not crosslinked or attached to the capillary wall</td>
</tr>
<tr>
<td>• Well-defined pore structure</td>
<td>• Entangled polymer networks of linear or branched hydrophilic polymers</td>
</tr>
<tr>
<td>• Pore size cannot be varied after polymerization</td>
<td>• Dynamic pore structure</td>
</tr>
<tr>
<td>• Heat sensitive</td>
<td>• Pore size can be varied</td>
</tr>
<tr>
<td>• Particulates can damage gel matrix</td>
<td>• Heat insensitive</td>
</tr>
<tr>
<td>• Provide extremely high resolution (oligonucleotides)</td>
<td>• Particulates can be easily removed</td>
</tr>
<tr>
<td>• Not replaceable; generally high viscosity</td>
<td>• Gel is replaceable when a relatively low-viscosity matrix is used</td>
</tr>
<tr>
<td><em>Example:</em> eCAP ssDNA 100 for oligonucleotide separations</td>
<td><em>Example:</em> eCAP dsDNA 1000 for PCR fragment analysis</td>
</tr>
</tbody>
</table>
Figure 11. Long-chain molecules cause a solution to become viscous (A) because they interfere with one another as the solution flows. As their concentration increases, the molecules become entangled, yielding a viscoelastic behavior that partakes of both solid and liquid traits (B). If the intertwined molecules bond with one another, the result is a crosslinked gel (C). Adapted with permission from “Intelligent Gels,” Osada, Y., and Ross-Murphy, S. B., Scientific American 268, 82 (1993). Copyright Scientific American, Inc. All rights reserved.
The chemical gels have a well-defined pore structure, they are rigid, and their pore size can be varied by adjusting the polymerization conditions (e.g., by varying the monomer and crosslinker concentration ratio in the PA gels), as is well known from the practice of classical electrophoresis. As shown below, with these type of gels in CE, extremely high-resolution separations of oligonucleotides can be achieved (e.g., Guttman et al., 1990; Schomburg, 1993).

The non-crosslinked, replaceable polymer networks have a dynamic pore structure and are more flexible. Polymer networks of variable viscosity can be made by carefully selecting the concentration and chain length of the linear polymers. With CE, solutions consisting of linear PA, various alkylcelluloses, low-melting agarose, and other polymers have been employed (see Section 4.2.3 for a listing). Conceivably, many other polymers are also suitable to serve as gel media for CE.

It is interesting to note that very dilute polymer solutions (≈ 0.010 to 0.001% hydroxyethylcellulose) are still effective in size-separating DNA (Barron et al., 1994). A fully entangled polymer network does not appear to be a prerequisite for separation. A mechanism other than the Ogston and reptation models (Figure 6) may be operative with DNA electrophoresis in entangled polymer solutions. As envisioned by Soane and co-workers (Barron et al., 1993 and 1994), when DNA migrates through a polymer network, entanglement coupling between the DNA and the surrounding polymer chains occurs, as schematically shown in Figure 12. Ultimately, this coupled entanglement is thought to limit the resolution achievable with relatively large DNA fragments (Bae and Soane, 1993).

Figure 12. A schematic representation of the entanglement coupling interaction of DNA with the polymer chains of the sieving matrix. Reproduced with permission from Barron et al., J. Chromatogr. A 652, 3 (1993).
An important advantage of the physical gels is that a low enough viscosity can be selected so that the contents of the capillary can be rinsed in and out of the capillary (in P/ACE by 20 psi positive pressure). As mentioned before, these gels are often referred to as replaceable matrices. When desired, therefore, a fresh gel can be used for every sample injection. In addition, sample introduction is possible by either the pressure or the electrokinetic mode, in contrast to the chemical gels (and high-viscosity polymer networks) where only the electrokinetic mode is possible (see also Section 4.2.7). This advantage of having the pressure injection mode available can be important in work dealing with quantitation. With polymer solutions, CE can be performed at relatively high temperatures (50 to 70°C) and field strengths (1000 V/cm) without damaging the gel as would be the case with chemical gels such as crosslinked PA gels.

Beckman offers three, linear-PA-based capillary gel kits; their features are described in Table 3. Gel-filled capillaries recently have been reviewed by Baba and Tsuhako (1992), Guttman (1994), Schomburg (1993), and Pariat et al. (1993).
<table>
<thead>
<tr>
<th>Feature</th>
<th>eCAP ssDNA 100 Kit</th>
<th>eCAP dsDNA 1000 Kit</th>
<th>LIFluor™ dsDNA 1000 Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel Format</td>
<td>Fixed, polyacrylamide/urea-containing gel</td>
<td>Replaceable entangled polymer</td>
<td>Replaceable entangled polymer</td>
</tr>
<tr>
<td>Detection Method</td>
<td>UV or LIF</td>
<td>UV</td>
<td>UV or LIF</td>
</tr>
<tr>
<td>Nucleic Acid Type</td>
<td>RNA and single-stranded DNA</td>
<td>Double-stranded DNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>Size Range</td>
<td>Optimal resolution up to 100 bases</td>
<td>Molecular weight linearity 100 to 1000 base pairs</td>
<td>Molecular weight linearity 100 to 1000 base pairs</td>
</tr>
<tr>
<td>Resolution</td>
<td>Single base up to 100 bases</td>
<td>5–15 base pairs typical for fragments &lt; 400 base pairs</td>
<td>5–15 base pairs typical for fragments &lt; 400 base pairs</td>
</tr>
<tr>
<td>Injection Technique</td>
<td>Electrokinetic</td>
<td>Hydrodynamic for quantitation without sample preparation; Electrokinetic for maximum sample loading</td>
<td>Hydrodynamic for quantitation without sample preparation; Electrokinetic for maximum sample loading</td>
</tr>
<tr>
<td>Minimum DNA Concentration</td>
<td>≈ 7 ng DNA/mL</td>
<td>≈ 500 ng DNA/mL</td>
<td>≈ 1 ng DNA/mL</td>
</tr>
<tr>
<td>Detection Limits (On Column)</td>
<td>Not determined</td>
<td>≈ 150 attomoles per fragment by UV detection</td>
<td>≈ 300 zeptomoles per fragment by LIF detection</td>
</tr>
<tr>
<td>Applications</td>
<td>Primer/probe purity and antisense DNA purity</td>
<td>PCR product and RFLP analysis</td>
<td>PCR product and RFLP analysis</td>
</tr>
</tbody>
</table>
4.2.1 Polyacrylamide

The polymerization of crosslinked and high-viscosity (i.e., > 6%T), linear polyacrylamide (PA) is carried out within the fused-silica capillary tubing. For reasons of stability, the PA gel must be covalently bound to the wall of the column, e.g., by means of a bifunctional agent such as (3-methacryloxypropyl)-trimethoxysilane. After completion of this pretreatment procedure (Guttman, 1994), the polymerization reaction can take place in the capillary.

Crosslinked PA gels are used mainly for ss oligonucleotide separations of up to 200 bases, usually under denaturing conditions of 7 to 9 M urea (Section 5.2). The typical gel concentration is 3–6%T with 3–5%C. The resolving power depends on the length of the capillary (20 to 150 cm). Longer columns give higher resolution at the expense of longer separation times. Figure 13 illustrates the ultra-high resolving power feasible with these types of gel capillaries. The 160-mer in the lower trace of Figure 13 has a plate count of 30 million plates per m, while the peak width is only a few seconds! The high resolving power of the crosslinked gels is the reason why they often have been used in capillary DNA sequencing (see also section 5.3). However, medium-viscosity, linear PA gels (3–6%T) are easier to work with and can be replaced by pressure rinsing of the CE instrument.

Denaturing and the non-denaturing systems can be used with PA gel capillaries. Denaturing PA gel-filled capillary columns are utilized mainly for size separation of short ssDNA (up to several hundred bases, e.g., DNA primers and probes), and in DNA sequencing. The most commonly used denaturing agent is urea, while formamide is useful in some applications (Ruiz-Martinez et al., 1993). Beckman offers a kit for oligonucleotide analysis (eCAP ssDNA 100) which contains prepacked, gel-filled capillaries, Tris-borate buffers, 7 M urea and standards. Figure 14 illustrates the high resolving power of the eCAP ssDNA 100 column using the normal P/ACE configuration (injection at the long end of the capillary). Fast run times are possible by using the 7 cm, short end of the capillary with reverse sample injection (Figure 15). Non-denaturing PA gels may be useful when subtle differences based on the shape, size, and charge of the molecules are exploited (Guttman et al., 1992).
Figure 13. CGE of polydeoxythymidylic acid, p(dT)$_{20-160}$. The lower trace shows a blowup of the 24.0 to 24.8 min time interval, with the largest peak showing an efficiency of 30 million plates per m. Reproduced with permission from Guttman et al, Anal. Chem. 62, 137 (1990). Copyright: American Chemical Society.

Figure 14. Single-base separation of p(dA)$_{40-60}$ achieved using the eCAP ssDNA 100 Kit.
Figure 15. High-speed separation of $p(dA)_{25-30}$ accomplished using the 7-cm portion of the eCAP ssDNA 100 capillary.

The low-viscosity, linear PA polymer networks are not covalently bound to the capillary wall. In this case, the capillary is pre-coated with a suitable polymer, e.g., with linear PA which forms a monomeric layer on the inside of the capillary wall surface. With homemade replaceable capillaries, linear PA is generally used at low concentrations (1.5 to 6%). For the best polymerization reproducibility, it is recommended to prepare a high concentration gel (9 to 12%) that can be diluted to the appropriate concentration prior to use (Kleemiss et al., 1993; Guttman, 1994). Karger’s group at Northeastern University recently described the performance characteristics of the replaceable columns at various linear PA concentrations (Pariat et al., 1993). With a 6% linear PA capillary, the average peak efficiency was calculated as 4 million plates per m in the 51 to 267 bp region, making single bp resolution possible in this range. Comparisons of CGE with agarose slab gel electrophoresis for DNA digest analysis was done by Paulus and Husken (1993). CGE offered better resolution, especially in the < 600 bp range.

Non-denaturing, replaceable gel media are used (1.5 to 6% PA) for the separation of dsDNA molecules, such as restriction fragments or PCR products (see Section 5.4). Figure 16 shows the separation of a pBR322 DNA-$Msp$ I digest restriction fragment mixture using a replaceable PA gel matrix. The addition of a DNA intercalator to the run buffer (ethidium bromide in Figure 16) improves resolution (see also Section 4.2.4). Run-to-run reproducibility in these type of capillaries is excellent (typically $\approx 0.2\%$ RSD for migration times of DNA standards). Once conditioned, the replaceable capillaries can be used on a daily basis for months, e.g., for PCR product analysis.
Figure 16. Effect of ethidium bromide on the CGE separation of pBR322 DNA-Msp I digest restriction fragment mixture. (A) No ethidium bromide; (B) 1 mg/mL ethidium bromide in the gel–buffer system. Peaks (base pairs): 1 = 26, 2 = 24, 3 = 67, 4 = 76, 5 = 90, 6 = 110, 7 = 123, 8 = 147, 9 = 147, 10 = 160, 11 = 160, 12 = 180, 13 = 190, 14 = 201, 15 = 217, 16 = 238, 17 = 242. Conditions: (A) 100 V/cm, (B) 200 V/cm. Reprinted with permission from Guttman and Cooke, Anal. Chem. 63, 2038 (1991). Copyright: American Chemical Society.

4.2.2 Agarose

Agarose gels are characterized by large pore sizes, high mechanical strength, and biological inertness. As agarose is the medium of choice for the separation of relatively large DNA with slab gels, it seems logical that agarose would also be tried in capillaries. Compton and Brownlee (1988) showed preliminary results of DNA separations with agarose. Since then, only a few research groups (Bocek and Chrambach, 1991, 1992; Schomberg, 1993) have reported results with agarose-filled capillaries; they are not commercially available. Special purified grades of agarose must be used to avoid unwanted EOF in the capillary; in addition, agarose solutions must be optically clear for UV detection at 260 nm. Theoretical and practi-
cal aspects of DNA sieving in agarose have been published by Stellwagen (1987) and Upcroft and Upcroft (1993), respectively. DNA entanglement as it sieved through low-percent agarose was studied by Smisek and Hoagland (1990).

CGE of DNA restriction fragments with agarose concentrations between 0.3 to 2.6% at 40°C were described by Bocek and Chrambach (1991, 1992). The advantage of employing liquefied agarose above its “gelling” temperature is that the capillary is replaceable, i.e., it can be easily filled, rinsed, and refilled. Another advantage of this type of agarose is that its background absorbance at 254 to 260 nm is sufficiently low that DNA detection at the nanogram level is possible. With an agarose sieving matrix, the inner surface of the capillary must be coated with a suitable polymer, e.g., linear polyacrylamide. Using this technique, the effective size range for separation of dsDNA was limited to \( \approx 12 \text{ kb} \).

### 4.2.3 Alkylcellulose and Other Polymers

Apart from polyacrylamide and agarose, size separation of DNA in CE can be obtained by the use of various other entangled polymer solutions:

- hydroxyethylcellulose (HEC), e.g., Grossman and Soane, 1991; Nathakarnkitkool et al., 1992.
- hydroxypropylmethylcellulose (HPMC), e.g., Schwartz et al., 1991, 1992; Ulfelder et al., 1992; Baba et al., 1993.
- polyacryloylaminoethoxyethanol, e.g., Chiari et al., 1994; Nesi et al., 1994.
- ficoll-400, e.g., Righetti et al., 1991.
- polyethylene glycol, e.g., Zhu et al., 1989; Schwartz et al., 1991.
- glucomannan, e.g., Izumi et al., 1993.
- polyvinyl alcohol, e.g., Kleemiss et al., 1993.

As suggested by Soane and co-workers (Bae and Soane, 1993; Figure 12), a DNA fragment may be loosely entangled with matrix molecules as it is pulled through the solution by the electric field. The longer the DNA fragment, the more entanglement points will exist, and the slower the fragment will move through the capillary. Indeed, in CE of DNA, the electrophoretic mobility decreases with increasing fragment size (see equations in Section 2.3.1).
As with the PA-based sieving matrices, a precoated capillary is desirable with other sieving media. Commercially available polysiloxane coated capillaries (*e.g.*, DB-17 from J & W Scientific) can be used for this purpose, as well as others such as those coated with polyvinyl alcohol (Schomburg, 1993) or polyacrylamide (Strege and Lagu, 1991). It should be noted that the cellulose additives form an additional “dynamic” coating on the inner surface of the capillary wall—the EOF is significantly decreased (Schwartz *et al.*, 1991). DNA fragments will migrate in these coated capillaries in order of increasing size. (Note: if untreated capillaries are used in conjunction with alkaline buffer conditions, the reverse order of elution has been observed due to the effect of the EOF—see Grossman and Soane, 1991.)

The sieving of DNA through the medium can be manipulated by varying the chain length and the concentration of the polymer. This is illustrated in Figure 17 for a number of different polymers by plotting the mobility vs. the size (bp) of the DNA fragment on a semi-log scale. S-shaped curves—with a linear middle section—can be observed. The shallowness of the slope of the curve is a measure of the sieving power of the medium. The sieving depends on the viscosity of the medium and the polymer chain length. For example (panel A), the sieving is better at a higher concentration of the HPMC polymer (see also Figure 18). Comparison of panels A and B reveals that, at the same concentration, the short-chain polymer (HPMC-100) yields greater mobilities than the long-chain polymer (HPMC-4000), in agreement with earlier findings of Bode (1977). Panel C shows a steep curve for another sieving buffer consisting of 5% polyethylene glycol. It appears that this polymer solution would not be an effective sieving matrix for the DNA separations. Other publications dealing with the effect of polymer chain length are from Barron *et al.* (1993, 1994) and Baba *et al.* (1993).
Figure 17. Effect of different polymer additives on molecular sieving. The plots (semilogarithmic scale) show the dependence of mobility on the base pair number. DNA fragments from the HaeIII restriction digest of φX were used as base pair markers. Polymeric additives: (A) HPMC-4000 at 0.1, 0.35, 0.5, and 0.7%; (B) HPMC-100 at 0.5, 0.7, and 1.0%; (C) 5% PEG and polyacrylamide (3% T, 0.5% C). Reproduced with permission from Schwartz et al., J. Chromatogr. 559, 267 (1991).
Figure 18. Effect of the concentration of HPMC-4000 on the CE separation of the 1 kbp DNA ladder. Concentration of HPMC-4000: (A) 0.1%, (B) 0.3%, and (C) 0.7%. Reprinted with permission from Baba et al., J. Chromatogr. A 653, 329 (1993).

4.2.4 Intercalators as Buffer Additives

DNA-binding or intercalating dyes have been used for fluorometric DNA assays and in flow cytometry applications. Ethidium bromide (EB) was the first of such intercalators to be used for DNA assays (Le Pecq and Paoletti, 1967). Since then, a wide variety of even more sensitive dyes have been developed (see, for example, recent work by Glazer and Rye (1992).

Interestingly, the resolution of dsDNA separations in CE can be improved by using intercalating dyes (Schwartz et al., 1991 and Guttman and Cooke, 1991). This is usually done by adding dye to the running buffer (and/or sample) in concentrations of ≈ 0.5 to 5 µg/mL. The effect of EB on the separation of DNA restriction fragments was shown earlier in Figure 16. As shown in Figure 19, the dye molecule inserts itself (“intercalates”) between the base pairs of DNA, changing the molecular persistence length, conformation, and charge of the DNA. Since EB bears a positive
charge (opposite to that of DNA), the EB intercalation increases the migration times of all dsDNA fragments. Because of this complexation (approximately one EB molecule per five bp) and the increasing rigidity of the complex, the larger DNA molecules move relatively more slowly. Therefore, the separation time window widens, which increases peak capacity. Intercalators such as EB are, therefore, useful for manipulating migration time and separation. For example, it is even possible to achieve baseline separation of dsDNA species that have an identical chain length but are composed of a different sequence (see the two 147 bp fragments and the two 160 bp fragments in Figure 16).

Figure 19. The intercalation of ethidium bromide into a DNA molecule. Ethidium bromide increases the spacing of successive base pairs, distorts the regular sugar–phosphate backbone, and decreases the pitch of the helix. Reproduced with permission from Watson et al., Molecular Biology of the Gene, Menlo Park, CA, Benjamin/Cummings Publishing Company, 1987.
It is important to note that the intercalating dyes strongly fluoresce when excited with an appropriate light source. This opens up the possibility of using (laser-induced) fluorescence detection with CE. As will be discussed in Section 4.3, extremely sensitive LIF detection methods can be utilized for tracing minute amounts of DNA.

4.2.5 Ferguson Plots

The size selectivity of sieving media (e.g., PA slab gels) is often characterized by “Ferguson” plots (Andrews, 1986; Stellwagen, 1987). Figure 20, from Heiger et al. (1990), shows the PA gel concentration (expressed as %T) vs. the log mobility for linear PA in a CE system. The larger the DNA fragment, the steeper the slope, in accordance with the sieving theory (see Equation 6, Section 2.3.1). From the intercept of the plot, the free solution mobility (i.e., in the absence of a gel matrix) of a DNA fragment can be calculated. From Figure 20 it also appears that the mobility of the DNA fragments in free solution, at 0%T, is virtually independent of the size of the DNA fragments examined. Schwartz et al. (1991) showed similar Ferguson plots for sieving of DNA fragments in polymer networks of alkylcellulose.

![Ferguson plots for linear polyacrylamide. The lines represent the log mobility of various φX-174 HaeIII fragments as a function of monomer composition. Reprinted with permission from Heiger et al., J. Chromatog. 516, 33 (1990).](image-url)
4.2.6 Instrument Parameters

When gel-filled capillaries are used for DNA separations, the power supply of the CE system must be in reverse-polarity mode i.e., with the cathode on the injection side and the anode on the detection side. DNA is monitored in real time by UV detection at 254 or 260 nm. When fluorescence or LIF detection is employed, the excitation and emission wavelengths depend on the fluor label or dye used. The temperature of the gel-filled capillary column is usually maintained at constant temperature during the experiments (± 0.1°C).

Separation of DNA restriction fragments can sometimes be improved by using different temperatures during the electrophoresis (Guttman and Cooke, 1991 B). Migration times decrease with increasing temperature in the isoelectrostatic (constant voltage) separation mode and maximize in the isorheic (constant current) separation mode. The resolution between the short DNA fragments (< 300 bp) decreases in the isoelectrostatic separation mode and shows maxima in the isorheic mode at elevated temperature. However, the efficiency in the higher MW range (> 1000 bp) decreases in both modes with increasing temperature.

Resolution and analysis time can also be optimized by manipulation of the electric field. Methods based on pulsed-field electrophoresis (Sudor and Novotny, 1994; Kim and Morris, 1994), analyte velocity modulation (Demana et al., 1991) and field-strength gradient separation techniques (Guttman et al., 1992 B) have been reported. The latter method can directly be performed with P/ACE; the first two require special instrumentation.

4.2.7 Sample Injection and Matrix Effects; Quantitation

4.2.7.1 Replaceable Gels

Quantitation. With replaceable gels (e.g., the eCAP dsDNA 1000 from Beckman), both pressure (typically: 2 to 20 sec, 0.5 psi) and electrokinetic injection are feasible. In CE, the pressure injection mode is generally recommended for quantitative work: the composition of the sample plug introduced into the capillary is exactly that of the sample vial from which the injection took place. In addition, sample preparation is simplified as no desalting needs to be performed. Butler et al. (1994) recently reported precision results with replaceable gels. Using pressure injection and an internal standard, peak migration time precision was < 0.1% RSD, whereas
the area precision was \( \approx 3\% \) RSD (Table 4). It should be noted, however, that electrokinetic injection often yields more efficient peaks than does pressure injection (Schwartz et al., 1991; Butler et al., 1994). When electrokinetically injected from low-ionic-strength sample solutions, DNA fragments are effectively stacked against the relatively viscous, polymer network medium. In separations of small molecules, electrokinetic injection may give rise to a sampling “bias” as sample components, because of their different mobilities, move into the capillary at different speeds. However, since DNA fragments essentially have the same mass-to-charge ratio in free solution, no such sample bias occurs when these fragments are electrokinetically injected from an aqueous solution.

<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 4. Peak Area Precision (RSD) with Replaceable Gels</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Hydrodynamic</th>
<th>Electrokinetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Internal Standard</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted Area</td>
<td>8.0%</td>
<td>6.0%</td>
</tr>
<tr>
<td>Height</td>
<td>8.1%</td>
<td>3.0%</td>
</tr>
<tr>
<td>Migration Time</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>(B) No Internal Standard</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted Area</td>
<td>8.4%</td>
<td>28%</td>
</tr>
<tr>
<td>Height</td>
<td>8.5%</td>
<td>23%</td>
</tr>
<tr>
<td>Migration Time</td>
<td>0.2%</td>
<td>0.3%</td>
</tr>
<tr>
<td>(C) Internal Standard</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted Area</td>
<td>3.0%</td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>6.7%</td>
<td></td>
</tr>
<tr>
<td>Migration Time</td>
<td>0.07%</td>
<td></td>
</tr>
</tbody>
</table>

(A) 100 bp DNA compared to 200 bp DNA internal standard for 10 runs. Both inlet and outlet run buffer vials were changed after run 5. (B) Without using internal standard. (C) Same as (A) but outlet buffer vial was changed after every run.

**Matrix Effects; Desalting.** In the practice of CE, separation performance is often strongly dependent on the composition of the sample solution. This is particularly important when samples are electrokinetically injected and variable amounts of salt are present. Desalting the sample by ultrafiltration—in conjunction with electrokinetic injection—may enhance sample detectability, as demonstrated by Schwartz et al. (1991), Ulfelder et al. (1992), and Butler et al. (1994). The ultrafiltration procedure removes low-
MW sample constituents, resulting in efficient DNA peaks. Figure 21 shows the dramatic effect of desalting the sample for two co-amplified PCR products from an HIV-1-positive control cell line—no peaks are visible in the trace corresponding to the untreated sample. However, it has been reported that desalting—when used in conjunction with pressure injection—may also lead to loss of DNA due to adsorption on the filter (Butler et al., 1994). Thus, when possible, pressure injection—without desalting—is preferable.

**Figure 21.** Effect of ultrafiltration on the PCR-amplified DNA peaks. (A) Untreated sample (no ultrafiltration), a co-amplified HIV-1, HLA-positive (115 and 242 bp, respectively) control. (B) Desalted sample. Adapted with permission from Schwartz et al., J. Chromatogr. 559, 267 (1991).

Recently, van der Schans et al. (1994) studied sample matrix effects for analysis of PCR products with replaceable gels and pressure injection. When the sample plug length was increased, decreased efficiency—apparent as fronting peaks—was observed. Sharpening of the peaks can be obtained by simply injecting a plug of low resistance, 0.1 M Tris-acetate prior to the sample injection (Figure 22). The lower field conditions existing in the Tris-acetate plug cause electrophoretic stacking of DNA fragments.

Co-injection of PCR products with a standard is a convenient method of verifying the identity of the sample peaks. It was found that co-injection of a DNA standard with the PCR sample can lead to sharpening of the sample peaks or the standard peaks, depending on the order in which the plugs were loaded on the capillary. This effect is shown in Figure 23. In the top trace, the 97 bp PCR sample is injected first, followed by the φX-174 HaeIII DNA standard. It can be seen that, while the standard peaks
are sharp, the PCR peak is broadened. The opposite is seen in the lower trace where the injection order was reversed. The broadened peaks are due to salt migrating from the PCR sample into the plug containing the DNA standards. During its migration through the capillary, the back end of the standard zone will migrate at a slower velocity relative to the front as here the field strength is lower than at the front end of the plug.

Figure 22. (A) Electropherogram of a 50 mg/mL φX-174 RF DNA HaeIII dissolved in 20 mM NaCl. (B) Influence of presample injection of 0.1 M Tris-acetate, pH 8.3. Injection procedure: first injection: 10-s pressure injection of Tris-acetate; second injection: 20-s pressure injection of 50 mg/mL φX-174 HaeIII sample in 20 mM NaCl. Reprinted with permission from van der Schans et al., J. Chromatogr. A 680, 511 (1994).
Figure 23. (A) Electropherogram of PCR sample and DNA standard. First injection: PCR sample (97 bp); second injection: φX-174 HaeIII 10 mg/mL. (B) Electropherogram of DNA standard and PCR sample. First injection: φX-174 HaeIII 10 mg/mL; second injection: PCR sample (97 bp). Reprinted with permission from van der Schans et al., J. Chromatogr. A 680, 511 (1994).

4.2.7.2 Non-Replaceable Gels

When working with capillaries containing high-viscosity gels (e.g., > 6% linear PA), sample introduction can only be performed by electrokinetic means (typically 0.015 to 0.15 Ws is applied). This is the case for the eCAP ssDNA 100 column from Beckman, as well as with capillaries containing highly viscous alkylcellulose or similarly entangled polymer networks. Pressure injection is limited with highly viscous media; the volume of the sample injected is inversely proportional to the viscosity of the
buffer. Exceedingly long injection times would be required, making pressure injection impractical.

When using the eCAP ssDNA 100 capillary and electrokinetic injection mode for quantitative studies, effects due to the presence of salts or other substances in the sample matrix must be carefully considered. For example, when electrokinetic injections are made from samples containing different salt concentrations, the amount of analyte introduced into the capillary will vary. This, in turn, has consequences for the accuracy of a drug assay. As noted by Srivatsa et al. (1994), with many pharmaceuticals for intravenous or ophthalmic use, the products are formulated in isotonic salt solutions. In this case, it is important to use an external reference standard with the same sample matrix in order to accurately assay a drug product. In CGE with electrokinetic injection, while the peak migration time precision generally is excellent (< 0.2% RSD), peak area precision may exceed tolerable levels. Precision can be greatly improved, however, by the use of an internal standard (IS), as shown in Table 5 for a series of electrokinetic injections of an antisense DNA oligonucleotide (ISIS 2922) and its N-1 deletion sequence on an eCAP ssDNA 100 capillary.

| Table 5. Reproducibility of Integrated Peak Area |

<table>
<thead>
<tr>
<th>Observed Peak Area Normalized to IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISIS 2922</td>
</tr>
<tr>
<td>0.168487</td>
</tr>
<tr>
<td>0.111345</td>
</tr>
<tr>
<td>0.154746</td>
</tr>
<tr>
<td>0.162939</td>
</tr>
<tr>
<td>0.196860</td>
</tr>
<tr>
<td>0.115623</td>
</tr>
<tr>
<td>0.104393</td>
</tr>
<tr>
<td>0.281972</td>
</tr>
<tr>
<td>0.362852</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Std. Dev.</td>
</tr>
<tr>
<td>% RSD</td>
</tr>
</tbody>
</table>
4.2.8 Hybridization; Southern Blotting, Mobility Shift Assays

In Southern blot hybridization, typically a slab gel is run to separate DNA fragments digested from nuclear DNA (Southern, 1975). After blotting the fragments to a membrane, a hybridization reaction is carried out to identify particular nucleotide sequences. When used in conjunction with autoradiography, this can be an extremely sensitive method (for example in forensic applications—see Figure 5). It is also a very selective method, as the target DNA will only bind with probe DNA that has a complementary sequence. However, the classical method is laborious and time consuming, often taking several days to complete. Brownlee, Sunzeri, and co-workers (1990, 1991) were the first to demonstrate the feasibility of hybridization with fluorescently labeled probes for the detection of target DNA (HIV-1 and HTLV-1) sequences. Using rhodamine and FITC-labeled probes, these sequences could be discriminated in a single run by fluorescence diode-array detection at 563 nm and 519 nm, respectively. Also working with HIV-1 genome sequences, Bianchi et al. (1993) analyzed PCR products by pre-capillary hybridization. A 299-nucleotide ssDNA fragment was hybridized with a complementary 28-mer, resulting in mobility shifts in the electropherogram.

Cohen and co-workers have shown that it is possible to transfer Southern blotting from a slab gel to a CGE format, thereby greatly reducing the analysis time. In their first paper (Cohen et al., 1991), preliminary results were reported with probes labeled with the fluorescent dye, Joe. Analysis was made by LIF detection, using a 488 nm Ar-ion laser. In a later paper (Vilenchik et al., 1994), antisense DNA (phosphorothioate) was quantified as low as 0.1 ng/mL using fluorescein-labeled probes. Although UV detection also was used, LIF detection (488 nm, Ar-ion laser) gives superior sensitivity. In addition, background DNA in the detector or sample, which is problematic with UV detection, does not affect LIF detection. The principle of the CE method used by Cohen and co-workers is illustrated in Figure 24 (UV traces). Sample preparations containing different amounts of target and probe (kept constant here) were injected onto two capillaries: one under non-denaturing CGE conditions (set A), the other under denaturing CGE conditions (set B). The electropherograms show three peaks: that of the target antisense DNA (“GEM”), that of the probe (“COM”), and that of the hybrid (“duplex”). As the GEM concentration increased, the duplex peak also increased in size, while the COM peak size decreased. In the set of denaturing electropherograms, the duplex peak—as expected—is not
observed. The order of migration of the target and the probe is different than that under non-denaturing conditions, most likely due to secondary structure differences between the two.

**Figure 24.** The separation of GEM, COM, and duplex by CGE. Electropherograms 1, 2, and 3 show different amounts of GEM with constant COM concentration. (A) Non-denaturing conditions. (B) Denaturing conditions. Conditions: (A) 9%T linear polyacrylamide column, effective length = 20 cm, applied electric field = 200 V/cm; (B) 13%T linear polyacrylamide, denaturing conditions, effective length = 15 cm, applied electric field = 400 V/cm. Adapted with permission from Vilenchik et al., J. Chromatogr. A 663, 105 (1994).
LIF detection of the antisense DNA proved to be quantitative and linear over three orders of magnitude. However, in the CE-LIF electropherograms, the fluorescein-labeled probe co-eluted with the duplex (as opposed to the UV traces where resolution is adequate, Figure 24). Using ethidium bromide (0.04 µM) as an intercalating dye, it was possible to increase the resolution sufficiently to enable quantitative analysis (see also Section 4.2.4).

CE can be used as a tool to study biomolecular, non-covalent reactions. The term affinity capillary electrophoresis (ACE) has been coined to describe the CE of receptor-ligand interactions, including those of antigen-antibody (immunoassays). A number of recent papers have demonstrated the potential of ACE for DNA studies. For example, Heegaard and Robey (1993) used ACE for the study of oligonucleotide-peptide interactions. Using dimeric peptides as probes, the binding was found to depend on both the size of the oligonucleotide and the specificity of the interaction. In another paper, Rose (1993), using CGE (high-viscosity gel), studied the binding kinetics of a type of antisense DNA (peptide nucleic acids, PNAs) with oligonucleotides. In PNAs, the deoxyribose-phosphate backbone is substituted for a peptide backbone. The free PNA, oligonucleotide, as well as the bound heteroduplexes were efficiently separated and quantitated.

Protein-DNA interactions have also been studied with CE. These interactions are involved in control of replication, recombination, modification, repair, and transcription processes. Methods for studying DNA-protein interactions include mobility shift assays, where slab gel electrophoresis is used to detect a change in mobility of DNA when complexed to a protein. CE can be applied to these types of mobility shift assays, as shown by Maschke et al. (1993) for the binding of an endonuclease, EcoRI, with oligonucleotides. A free-solution CE system with LIF detection (Ar-ion laser, 488 nm) was used. Joe-labeled oligonucleotides with the EcoRI recognition site, GAATTC, interact with the protein; the complex is detected as a faster-migrating fluorescent peak. Addition of excess unlabeled probe displaces the labeled probe in the complex, resulting in the disappearance of the fluorescent signal.
4.3 Detection in CE: UV Absorbance vs. Laser-Induced Fluorescence (LIF)

Optical detection techniques for CE recently have been reviewed by Pentoney and Sweedler (1994). In the vast majority of DNA as well as other applications of CE, UV-Vis absorbance detection has been used. Practically all commercial CE instruments are equipped with this detector, which is universal (i.e., suitable for many types of analytes) and also has adequate sensitivity for most applications. However, in bioscience applications, often trace amounts of an analyte need to be determined in the presence of many other sample components, and detection may become problematic. The detection limit with UV detection is—among other factors—related to the small interior diameter of the capillary; for example, a 200 bp DNA fragment typically has a minimal detectable concentration of \( \approx 0.5 \mu g/mL \). Optimal stacking and/or ITP preconcentration methods, as well as optimized optics, may improve the detection limits by a factor of 2 to 10. Fluorescence detection, however, may yield far lower detection limits and has been used for decades with many DNA applications. Fluorescence-based assays have the advantage of offering both excellent selectivity and very high sensitivity.

Fluorescence or LIF-based detection, in conjunction with fluor labeling systems, has been developed for many biomedical applications, e.g., chromosome sorting, DNA sequencing and fingerprinting. With sophisticated instrumentation, it has become possible to examine the protein or nucleic acid content of single human cells or even to detect single molecules of stained DNA. Recently, an LIF detector has been developed by Beckman for CE. Figure 25 shows a detail of the optical design used in the P/ACE 5000 Capillary Electrophoresis System.
Figure 25. Schematic view of a CE-LIF detector (Beckman Instruments, Inc., Fullerton, CA). Fiber-optic cable transmits laser light from the laser to the detector and illuminates a section of the capillary. Fluorescence is collected by the ellipsoidal mirror and focused back onto the photomultiplier tube. To reduce unwanted laser light, a centered hole in the mirror allows most of the beam to pass. A beam block is used to attenuate scattered laser light.

The P/ACE-LIF interface is supplied with a 488 nm Ar-ion laser but can be connected to various other laser sources. The detector incorporates an ellipsoidal reflector to maximize the emission light collection efficiency. Table 6 shows a list of laser sources and excitation lines which have been used in some CE-LIF applications. Thus far, most work in CE has been performed with the easy-to-use and relatively low-cost Ar-ion, He-Cd, and He-Ne lasers. These laser sources are also well suited for DNA and nucleotide work. The 488 nm emission of the Ar-ion laser matches the 490 nm peak of popular fluorescein-based labels (FITC, fluorescein succinimidyl ester). The 325 nm emission of the He-Cd laser matches OPA and dansyl labels. The compact and even less expensive “green or yellow” He-Ne lasers can be used with various rhodamine derivatives and are compatible with intercalating dyes such as ethidium bromide or propidium bromide (Kim and Morris, 1994 B; Liu et al., 1995). Of all the laser sources, the mixed Ar-Kr lasers provide the widest wavelength range available (more than 20 emission lines in the 350 to 725 nm region).
### Table 6. Examples of Laser Light Sources for Detection with CE

<table>
<thead>
<tr>
<th>Laser Source</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar-ion (air cooled)</td>
<td>457, 472, 476, 488, 496, 501, 514</td>
</tr>
<tr>
<td>Ar-ion (full frame)</td>
<td>275, 300, 305, 333, 351, 364, 385, 457, 472, 476, 488, 496, 501, 514</td>
</tr>
<tr>
<td>Ar-ion (full frame, frequency doubled)</td>
<td>229, 238, 244, 248, 257</td>
</tr>
<tr>
<td>Ar-Kr</td>
<td>350-360, 457, 472, 476, 488, 496, 501, 514, 521, 531, 568, 647, 752</td>
</tr>
<tr>
<td>He-Cd</td>
<td>325, 354, 442</td>
</tr>
<tr>
<td>He-Ne</td>
<td>543, 594, 604, 612, 633</td>
</tr>
<tr>
<td>Excimer</td>
<td></td>
</tr>
<tr>
<td>XeCl (pulsed)</td>
<td>308</td>
</tr>
<tr>
<td>KrF (pulsed)</td>
<td>248</td>
</tr>
<tr>
<td>Nitrogen (pulsed)</td>
<td>337</td>
</tr>
<tr>
<td>Nitrogen pumped dye (tunable)</td>
<td>360-950</td>
</tr>
<tr>
<td>Solid state</td>
<td></td>
</tr>
<tr>
<td>YAG (frequency doubled)</td>
<td>532</td>
</tr>
<tr>
<td>YAG (frequency quadrupled)</td>
<td>266</td>
</tr>
<tr>
<td>Diode lasers</td>
<td></td>
</tr>
<tr>
<td>frequency doubled (LiNbO3)</td>
<td>415</td>
</tr>
<tr>
<td>frequency doubled (KTP)</td>
<td>424</td>
</tr>
<tr>
<td>frequency tripled (Nd-doped YLiF)</td>
<td>349</td>
</tr>
</tbody>
</table>

Low-cost, semiconductor (“diode”) lasers can also be used with CE. Their wavelengths are in the 635 to 850 nm range; background fluorescence from biological sample matrices is strongly reduced at these long wavelengths. The analytically relevant blue light (i.e., wavelengths compatible with the popular fluor labels) can be obtained from these lasers by
“frequency doubling” techniques of (infra)red light. In order to extend the applicability of the red diode lasers to CE, new labeling reagents (based on thiazine-, oxazine-, and cyanine-type compounds) are being developed, because just a few analytes yield native fluorescence in the red or near-IR region (Jansson et al., 1993). At Beckman Instruments, Inc. (Chen et al., 1993), a cyanine dye (Cy5) has been evaluated for potential use in DNA hybridization and sequencing (see also Section 5.3, Figure 35).

4.3.1 DNA Detection with LIF

4.3.1.1 Native and Indirect Fluorescence

A number of detection schemes have been used for the detection of nucleic acids by fluorescence methods. One approach is based on the native DNA fluorescence in the low-UV region (Milofsky and Yeung, 1993). Native fluorescence allows analysis of the DNA molecule in its “natural” state, with excimer lasers (e.g., the pulsed KrF, 248 nm laser) as the excitation source. A later paper (McGregor and Yeung, 1994) describes some improvement in detectability (lower background signal) with this approach by using a sheath-flow arrangement while separations were performed at pH 2.8.

Another LIF detection scheme for DNA is based on indirect fluorescence. A fluorogenic CE buffer system such as salicylate can be used in conjunction with laser excitation (e.g., the 325 nm He-Cd laser). Examples of nucleotides (Kuhr and Yeung, 1988) and dsDNA (Chan et al., 1993) have been demonstrated using this approach. However, the methods based on native and indirect fluorescence have not been widely used by other workers and special, homemade instrumentation is required.

4.3.1.2 Intercalators

The most straightforward and currently popular LIF detection scheme for CE involves the use of fluorescent intercalators (Schwartz and Ulfelder, 1992). Beckman has introduced a CE kit for dsDNA analysis which incorporates a specific intercalator dye, EnhanCE. The nuclear stain is added to the CE buffer and/or sample and specifically interacts with sample dsDNA or RNA molecules (see also section 4.2.4). The dye inserts itself between the base pairs of DNA, thereby changing its persistence length, conformation, and charge, resulting in a reduction of electrophoretic mobility but providing enhanced resolution (Figure 26). More importantly, the DNA-dye complex fluoresces strongly when excited by the appropriate laser,
whereas the intercalator alone—as well as non-DNA sample components—generally do not. Hence, separation selectivity is often vastly improved giving rise to a much “cleaner” electropherogram than possible with UV detection (see section 5.4.1, Figure 38).

![Figure 26. A plot of the resolution capability for both the eCAP and LIFluor dsDNA 1000 Kits, expressed as base pairs resolved for a specific DNA fragment size.](image)

The use of fluorescent intercalating dyes leads to 2 to 3 orders of magnitude enhanced sensitivity when compared to UV detection (Schwartz and Ulfelder, 1992; Ulfelder, 1993; Rossomando et al., 1994; Zhu et al., 1994; Arakawa et al., 1994 B). This is shown in Figure 27 for φX-174 HaeIII RF DNA fragments: the UV trace shows an appreciable noise level whereas, in the LIF trace, a “clean” baseline is obtained with no visible noise. In addition, the DNA concentration used for the UV detection was 20 times higher than that of the LIF.
Figure 27. Separation of an HaeIII restriction digest of \( \phi X \) 174 RF DNA using (A) UV and (B) LIF detection. Samples were diluted in water to a total DNA concentration of 200 \( \mu \)g/mL for UV detection; 10 \( \mu \)g/mL for LIF detection. Injection was by pressure to ten seconds. Buffer systems were the same, except for the addition of Enhance for LIF detection. From Ulfelder, K. J., Beckman Application Information Bulletin A-1748 (1993).
Monomeric as well as dimeric dyes have been utilized in recent CE-LIF applications (Zhu et al., 1994; Kim and Morris, 1994 B; Figeys et al., 1994). Ethidium bromide, in conjunction with a green He-Ne laser, can also be used for sensitive LIF detection (Liu et al., 1995). The detection of PCR products will be discussed later in Section 5.4. The 488 nm Ar-ion laser incorporated in the P/ACE-LIF instrument is compatible with many of these DNA and RNA dyes. It has been reported that the dimeric dyes (e.g., ethidium homodimer, TOTO, YOYO) are best used in conjunction with monomeric intercalator additives, e.g., 9-aminoacridine (Zhu et al., 1994); otherwise, broad peaks may result from the presence of multiple dye-DNA bonding.

4.3.1.3 Fluorescent Labeling

The final LIF detection scheme for DNA involves direct labeling of the analyte with a suitable fluorophore. Fluorescently labeled probes and primers are used in many molecular biology applications involving hybridization and PCR (Mansfield and Kronick, 1993). DNA primers and probes are usually synthesized with a fluorescent label attached to the 5' end of the molecule, or with post-synthesis attachment of a dye using commercial DNA labeling kits. Unincorporated dye and/or failure sequences are generally removed by LC methods. For subsequent use in the PCR leading to fluorescent DNA products, conditions can be optimized such that primer purification is not necessary. Figure 28 shows the UV and LIF traces of a fluorescein-labeled primer. While it appears in the UV trace that many impurities (failure sequences) are present, the LIF trace reveals that most of these are not labeled and therefore do not fluoresce. The large (N-1)-mer is due to a diasteriomer of the fluorescein phosphoramidite used in the labeling procedure (Ulfelder, 1994).
In DNA sequencing, fluorescently labeled primers based on fluorescein, modified fluoresceins, Texas Red, and tetramethylrhodamine are routinely used (Dovichi, 1994). The fluorescein dyes match the 488 nm line of an Ar-ion laser, whereas the rhodamine dyes are compatible with the 543.5 nm line of an He-Ne laser or the 514.5 nm line of an Ar-ion laser. For the labeling of nucleotides, fluors such as dansyl (Lee et al., 1991) and fluorescein (Li et al., 1993) have been used. Wang and Giese (1993) recently described a phosphate-specific labeling of nucleotides with the fluor BODIPY Fl C₃ hydrazide (Molecular Probes, Eugene, OR). The labeled nucleotides were subsequently detected by CE-LIF. Adenine-containing nucleotides were selectively analyzed by Tseng et al. (1994) using a chloroacetaldehyde derivatization reaction to convert the analytes to fluorescent products.
4.4 Fraction Collection: CE as a Micropreparative Tool

CE is not only an analytical technique but can also be used for micropreparative purposes. For example, it is possible to collect fractions from protein digests and subsequently perform microsequencing to identify the peptides. Enzyme activity in fractions of a fermentation broth can be determined using CZE in a micropreparative mode (Banke, 1991). In another DNA-related application, a small quantity (less than a µg) of a 20-mer oligonucleotide primer was collected by using micropreparative CGE; the collected fraction from a crosslinked gel (8%T, 3.3%C) was subsequently used in a dot-blot assay (Cohen et al., 1988). More recently, CGE (replaceable gel, 4% linear PA) was used to collect multiple peaks corresponding to denatured DNA from a mutated, 372 bp PCR product. The collected fractions were re-amplified by PCR and subsequently analyzed again by CGE. It was found that the different peaks corresponded to different gene sequences (Kuypers et al., 1993—see also Section 5.4.2.1).

4.4.1 Fraction Collection Using Field Programming

With automated instruments such as P/ACE, during a micropreparative run, the outlet of the capillary is switched from the buffer vial to a collection vial which contains a small amount (∼ 2 to 10 µL) of water or dilute buffer. (Note: relatively large-i.d. capillaries are beneficial in micropreparative CE as more material can be collected: the loadability of capillaries is proportional to their cross-sectional area). In the above-mentioned applications, the electric field was kept constant during the collection of the fractions. However, with CGE of DNA, often very narrow peak widths—a few seconds wide—are obtained. This makes reproducible collection of peaks difficult. By programming the electric field during the collection (“slowing the field down”), the collection process can be simplified (Guttman et al., 1990). This approach of field programming for fraction collection is demonstrated in Figure 29.
The goal of the experiment was to collect the 47-mer from a p(dA)$_{40-60}$ mixture. During the micropreparative run (trace A), the field was maintained at 300 V/cm until just before the 47-mer reached the end of the capillary. At that point, the field was decreased 10-fold and the fraction was collected for 60 s (the calculated peak width under the low-field conditions was 45 s). Trace B shows the reinjected collected fraction: only the 47-mer is visible together with the internal standard, a 20-mer. Typically, the micropreparative runs yield broader peaks than their corresponding analytical runs. The sample size injected for the micropreparative run resulting in the “overloaded” profile (trace A) was 6 times higher than the analytical run (trace C).

Figure 29. (A) Micropreparative CGE separation of a polydeoxyadenylic acid test mixture, p(dA)$_{40-60}$; (B) analytical run of the isolated p(dA)$_{47}$ spiked with p(dA)$_{20}$; and (C) analytical run of p(dA)$_{40-60}$ spiked with p(dA)$_{20}$. Reprinted with permission from Guttman et al., Anal. Chem. 62, 137 (1990). Copyright, American Chemical Society.
5 Selected Applications

5.1 Nucleotides, Nucleosides and Bases with CZE or MECC

Nucleotides and their nucleoside and base constituents play an important role in many vital biochemical processes. They are the activated precursors of DNA and RNA. Intracellular nucleoside metabolism is an important topic in AIDS research. A number of dideoxynucleoside analog drugs (azT, ddI and ddC) are currently used in the treatment of HIV-1-positive individuals or are in human clinical trials (d4T and ddA). The nucleoside “pro-drugs” are converted by intracellular host enzymes (kinases) into their triphosphates; the latter inhibit the viral reverse transcriptase enzyme. DNA “damage” at the nucleic acid base level is also actively studied (Ca-det and Weinfeld, 1993). Reliable, high-resolution analytical methods to quantitate nucleotides, nucleosides, and bases in biological samples (often at extremely low levels) are highly desirable in these areas. CE has been demonstrated to be a promising new tool in a number of recent studies. CE methods may complement existing HPLC methods; however, often superior resolution with shorter analysis time is possible with CE, while a minimal amount of sample is required for the process.

5.1.1 DNA Adducts; DNA Damage

DNA damaged by covalent modifications or additions of xenobiotic components was studied by Norwood et al. (1993) and Guarnieri et al. (1994). The first group evaluated different CZE and MECC conditions and demonstrated sample stacking techniques to increase detectability of benzo(a)pyrene-DNA adducts. Guarnieri et al. (1994) measured 8-hydroxydeoxyguanosine by MECC as a marker of DNA oxidation. Single-stranded DNA was incubated in the presence of an oxidizing agent and hydrolyzed by enzymatic digestion. MECC did not, however, permit determination of extremely low levels of oxidized nucleosides generated by endogenous sources of free radicals (see also Section 5.1.4).

5.1.2 Nucleoside Analog Drugs

Therapeutic drug monitoring of a nucleoside drug was described by Lloyd et al. (1991). The antileukemic agent, cytosine-β-D-arabinoside (Ara-C), was determined in human serum. The authors found low-level (i.e., sub-µM) detection of Ara-C problematic. However, by using solid-phase extraction
for concentration and sample cleanup, it was possible to determine Ara-C in the 1 to 10 µM range. This procedure removes most of the protein and allows doubling of the Ara-C concentration. The assay was validated over a concentration range of 1 to 10 µM. Response was linear in this range, with a correlation coefficient of 0.996 for the calibration plot. Compared to HPLC, the proposed assay has a rapid analysis time (no need to run a gradient to remove late eluting compounds) and is free from endogenous substances.

Rogan et al. (1993) showed that CE is well suited to resolve enantiomers of nucleoside analog drugs. Determining the enantiomeric ratio of such drugs is important to regulatory agencies such as the FDA because one enantiomer may exhibit far greater efficacy (or toxicity) than the other. For example, in the manufacturing process of an antiviral drug (Glaxo), the racemic 2’-deoxy-3’-thiacytidine (BCH-189) undergoes an enantiospecific deamidation to yield the (-) enantiomer. The latter chiral drug is less toxic and therefore preferred for clinical use over the racemic drug. The time course of the enzymatic reaction was followed by CE for more than two days (Figure 30). After 40 hours, less than 1% of the (+) BCH-189 remains. Dimethyl-β-cyclodextrin was added to the sodium phosphate, pH 2.3, run buffer as the chiral discriminator; baseline resolution was obtained between the (+) and the (-) enantiomers. Compared to HPLC methods which typically involve expensive chiral stationary phases, the CE approach is simpler, and reliability and precision reportedly are excellent (see the Beckman primer on chiral analysis, P/N 726388).

**Figure 30.** Time dependence of percentage of (+) enantiomer remaining during a biotransformation reaction time course. The two peaks represent the enantiomers of the nucleoside analog drug BCH-189. Electropherogram courtesy of Dr. K. Altria, Glaxo Group Research, U.K.
5.1.3 Nucleotides in Cell Extracts

Nguyen et al. (1990) used a CZE method for the quantitation of nucleotide degradation in fish tissues. In most fish, ATP degrades rapidly to IMP which, in turn, degrades to inosine and the base hypoxanthine. IMP gives fish a pleasant fresh taste while hypoxanthine accumulation results in an “off” taste. Tissue extracts were assayed by both CZE and an enzymatic method. Good correlation between peak area and nucleotide concentration was found. The CZE method involved UV detection, untreated, fused-silica capillaries, and a CAPS, pH 11, run buffer generating a high EOF.

Nucleotide profiles in cell extracts were determined by Ng et al. (1992) and Huang et al. (1990) with CZE. Ng et al. (1992) showed nucleotide profiles in human blood lymphocytes and leukemic cells. Reproducible areas and migration times were obtained using a P/ACE instrument. A simple CZE system using an untreated, fused-silica capillary with a borate, pH 9.4, run buffer was employed. The negatively charged nucleotides are carried to the detector (cathode) by the EOF. Fourteen of the common ribonucleotides were determined in a CZE assay by Huang et al. (1992). In this method, coated (polyacrylamide) capillaries resulted in negligible EOF using the mixed phosphate-Tris, pH 5.3, buffer. Electrophoretic flow carries the analytes to the detector end (anode, reversed polarity). Minimum detectable levels of the nucleotides were in the 1 to 10 µM range with UV detection at 254 nm. The method was applied to the quantitation of ribonucleotides in HeLa cells. In a later paper (Shao et al., 1994), Ucon-coated capillaries were used for the determination of ribonucleotides in lymphoma cells. A similar method, involving (polyacrylamide) coated capillaries, was described by Takigu and Schneider (1991). The authors discussed validation criteria, i.e., linearity and minimal detectable concentration (∼1 µg/mL per nucleotide without stacking). Beckman’s Neutral coated capillary (P/N 477441) is also suitable for these types of applications.

MECC conditions have also been proposed for the analysis of nucleotides in cell extracts. It appears that cationic surfactants are more effective than their anionic counterparts such as SDS. Perret and Ross (1991) selected dodecyltrimethylammonium bromide (DTAB), resulting in a charge reversal on the capillary wall as shown earlier in Figure 9. They also used 1 mM EDTA in the run buffer as a metal chelating agent to prevent metal-nucleotide interaction which was thought to result in peak tailing. Figure 31 shows that this method can be applied to acid extracts of cells. The upper trace shows the separation of a standard mixture of 15 nucleotides.
with a 50 mM phosphate (pH 7), 100 mM DTAB, 1 mM EDTA run buffer. A neutralized perchloric acid extract of rat tumor is shown in the bottom trace of Figure 31. The reported method gave a linear response up to 200 µM; migration time and peak area precision ranged from 2.2 to 5.5% and 3.3 to 6.1%, respectively. Similarly, Ramsey et al. (1994) evaluated a number of cationic surfactants for the MECC of nucleic acid constituents. Here, optimum resolution was achieved by using tetradecyltrimethylammonium bromide (TTAB) as the micellar reagent. Loregian et al. (1994) compared MECC with HPLC for the quantitation of ribonucleotide triphosphates in four different cell lines. The MECC method yielded approximately one million theoretical plates with detectability down to 50 fmol.

**Figure 31.** Separation of nucleotides by MECC. (A) Separation of standard mixture of 15 nucleotides at 25 mM each (B) neutralized perchloric extract of rat tumor. Adapted with permission from Perrett, Capillary Electrophoresis (Camillieri, Ed.), CRC Press, 1993.
5.1.4 Increasing Detectability: LIF Detection

There is increasing interest to measure nucleotides at extremely low levels in biological matrices or even in single cells. DNA base damage studies (by either HPLC or CE) are often hampered by a lack of sensitivity, especially when only limited sample volume is available. A sensitive assay should, for example, be capable of detecting at least one DNA base modification in $10^4$–$10^6$ normal bases within a few micrograms of DNA (Cadet and Weinfeld, 1993). CE with LIF detection is, in principle, sensitive enough to measure these types of modifications. Combined with suitable fluorophore chemistry, LIF detection can, in principle, provide orders of magnitude improvement in sensitivity over UV detection. Preliminary reports (Wang and Giese, 1993; Lee et al., 1991) on nucleotide analysis by CE-LIF support this contention.

5.2 Purity Control of Synthetic Oligonucleotides

Whereas the purity requirements may not be that important in applications where the oligonucleotide is used as a hybridization probe, in many other applications (including antisense DNA), the purity of the synthetic oligonucleotide needs to be ascertained. CGE is ideally suited for this purpose: the technique is fast, reproducible, has high resolving power, and does not involve radioactivity or toxic materials.

5.2.1 Phosphodiester Oligonucleotides

For the CE of oligonucleotides with 10 to 150 bases (primers or probes), crosslinked or linear polyacrylamide gels covalently bonded to coated, fused-silica capillaries are used under denaturing conditions (urea, formamide, heat). The prepacked, 100 µm-i.d. capillary available from Beckman (eCAP ssDNA 100) contains 7 M urea to prevent the formation of secondary structure of oligonucleotides. The capillary is designed for oligonucleotides in the 10 to 150 base range and can be used in the 20 to 50°C temperature range. An example illustrating the utility of this column for the QC of synthetic oligonucleotides was shown earlier in Figure 28.

The separation of a 119-mer oligonucleotide preparation on the eCAP ssDNA 100 column is shown in Figure 32. The upper trace shows the crude preparation; the lower trace shows the purified oligonucleotide. Separations of this type are difficult to perform with HPLC which generally is limited to an upper range of approximately 70 bases (Warren and Vella, 1993). In addition to the main component, the failure sequences are well resolved by the CGE capillary (N = 565,000 plates per m).
**Figure 32.** Analysis of a crude and purified synthetic 119-mer oligonucleotide using the eCAP ssDNA 100 Kit.

### 5.2.2 Antisense DNA

Antisense therapeutics are synthetic oligonucleotides that have a base sequence which is complementary to a target sequence on a messenger RNA (mRNA) which encodes for disease-causing proteins or to the double-stranded DNA from which the mRNA was transcribed. The complementary nature of the antisense molecule allows it to hydrogen bond and inactivate the genetic message, inhibiting gene expression. “Normal” oligonucleotides with a phosphodiester backbone are very susceptible to cellular nuclease degradation. There is, therefore, much interest in DNA analogs with phosphorus-modified backbones (*e.g.*, phosphorothioates and methylphosphonates, Figure 33) which exhibit increased resistance to these nucleases. Another type of antisense, termed peptide nucleic acid (PNA), in which the deoxyribose-phosphate backbone is substituted for a peptide backbone composed of (2-aminoethyl)glycine units, shows promise as a potent therapeutic agent and can also be analyzed by CGE (Rose, 1993). Because of their potential use as drugs, stringent purity requirements are typically required for antisense DNA agents.
CGE with the eCAP ssDNA 100 column from Beckman results in excellent resolution of deletion sequences of a 20-mer phosphorothioate antisense product. Figure 34 shows the electropherogram of a mixture of the n-mer and the (n-1)-mer. However, CGE cannot resolve phosphorothioates from their corresponding phosphodiesters (anion-exchange chromatography is the method of choice here) as the separation mechanism in CGE is based on molecular sieving (size). Using the eCAP ssDNA 100 Kit, Srivatsa, et al. (1994) demonstrated the validity of CGE for routine analysis of drug product formulations. The CGE method was found to be suitable for routine drug product analysis, as judged by several criteria, i.e., linearity, accuracy, selectivity, precision, and ruggedness—see also Section 4.2.7.2.
5.3 DNA Sequencing

Several research groups are currently exploring the use of CE as an alternative to slab-gel electrophoresis for automated DNA sequence determination (Pentoney et al., 1992; Ruiz-Martinez et al., 1993; Dovichi, 1994). The large surface-area-to-volume ratio of the capillary permits higher electric fields than are used typically with slab gels (due to more efficient heat dissipation), resulting in very rapid and efficient separation of sequencing reaction products. Additionally, the capillary format is readily adaptable to automated sample loading and on-line data collection. With CE, detection of separated DNA sequencing fragments is performed by LIF. The sensitivity of the LIF detection allows sequencing reactions to be performed on the same template and reagent scale as that of manual DNA sequencing with autoradiographic detection. The identity of the terminal base of each DNA sequencing fragment is encoded in the wavelength and/or the intensity of the fluorescent emission.

Sample throughput is a major concern for high-volume sequencing applications. Automation of sample preparation, sequence reactions (including electrophoresis), and data interpretation are necessary in order to
achieve the ambitious goal of sequencing the entire human genome (approximately 3 billion bp). With CE, the samples are loaded one at a time. Slab gels, on the other hand, can be simultaneously loaded with 24 to 36 samples. Instrumentation which would allow the running of several capillaries in parallel, together with robotics for sample handling, would dramatically increase the desired sample throughput with CE. Already DNA sequencing has been demonstrated in arrays of multiple (20 to 100) capillaries (Mathies and Huang, 1992; Ueno and Yeung, 1994; Takahashi et al., 1994). It can be easily envisioned that this type of instrumentation can also be incorporated in other applications, e.g., screening for genetic diseases, forensic DNA typing, etc.

A large obstacle to the development of commercial CE-based DNA sequencers has been the stability of gel-filled capillaries. While they can provide extremely high resolving power, the crosslinked gels typically last only a few runs when sequencing reactions are loaded, after which time the entire column must be replaced. Recent developments in CGE column technology (in particular, the replaceable gels) should eliminate the time-consuming and laborious procedures of the preparation and alignment of the capillaries (Ruiz-Martinez et al., 1993). With the replaceable matrix, it is possible to load a sequencing reaction, rapidly separate the DNA fragments at high field strength, and then reload the gel on the capillary prior to the next run. Figure 35 shows the CE separation of a single terminator, Sanger-Coulson reaction using a replaceable linear PA gel. A “red” diode laser was used for excitation of the fluor- (Cy5) labeled DNA fragments (Chen et al., 1993). The relatively low-viscosity (6%T) gel matrix of these types of capillaries provides reproducible and fast separation of DNA fragments with sequence information extending to at least 400 bases. For DNA sequencing applications, typically a CE run buffer containing formamide and/or urea is used. A denaturing buffer of 30% formamide, 3.5 M urea has a lower viscosity than a 7 M urea buffer and is therefore advantageous to use in a replaceable CGE formulation. In addition, increased decompression of sequences with secondary structures is obtained.
Figure 35. Electropherogram of fluorescently labeled (Cy5-20 primer) DNA fragments generated enzymatically (M13mp18 template) using ddG terminator. A LaserMax (Rochester, NY) red diode laser (639 nm excitation) was employed with an in-house-built CE-LIF instrument. The capillary was filled with a replaceable polyacrylamide gel. The pattern is recognizable beyond 400 bases. Electropherogram courtesy of Dr. S. L. Pentoney, Jr., Beckman Instruments (1994).


5.4 dsDNA, PCR Products Analysis (< 2000 bp)

With the advent of PCR-related methods, the number of publications involving DNA fragment separations by CE is rapidly expanding. Since its introduction in 1986, PCR technology is now used in a variety of diverse fields, e.g., medicine, biology, forensics, epidemiology, archeology, and nanotechnology, to name a few. Electrophoresis is almost invariably part of this research, i.e., as a tool to visualize DNA fragments as characteristic banding patterns which could reveal a disease gene in a patient, identify a suspect in a murder case, or establish part of the DNA sequence of a million-year-old fossil. In the years to come, CE, and especially CE combined with LIF, will undoubtedly replace classical electrophoretic techniques in many PCR-related applications.

The first researchers to investigate the utility of CE for dsDNA analysis were Brownlee, and co-workers at Microphoretic Systems (1988) and Cohen, Karger, and co-workers at Northeastern University (1988). From 1988 to 1991, emphasis was on the development of suitable gels/polymer networks and capillary coatings, and the fine-tuning of CE conditions to optimize separation performance (e.g., Zhu et al., 1989; Heiger et al., 1990; Guttman and Cooke, 1991 A; Schwartz et al., 1991). Applications, e.g., the screening of blood for HIV-1, were soon reported (Sunzeri et al., Brownlee et al., 1990). It also became apparent that detection by UV absorbance was not always sufficiently sensitive, e.g., in the detection of low copy DNA for viral screening (Mayer et al., 1991). Presently, the CE technology has advanced in this area and instrumentation (including high-sensitivity LIF detection) is commercially available. In the following sections, some selected applications dealing with the analysis of PCR products are highlighted. Arbitrarily, we have divided dsDNA analysis into two sections with the bp number smaller or larger than 2000.
Quantitation of Viral Load in Infectious Diseases

Quantitation of viral load in patient specimens is important to assess the stage of disease progression or to monitor the effectiveness of drug therapy. HIV-1 infection is a good case in point. Until recently, there were no reliable methods available to quantitate HIV-1 in the early-onset phase of infection, i.e., at low copy numbers. Novel PCR methods (e.g., competitive PCR) have recently allowed quantitation of proviral DNA or plasma viral RNA levels in patients with HIV-1 infection (Piatak et al., 1993) using gel electrophoresis with video scanning of the ethidium bromide-stained DNA. CE is ideally suited to replace slab gel techniques for these purposes. Competitive PCR is also adaptable to a CE format, as will be discussed in the next section.

Brownlee, Sunzeri, Mayer, and co-workers (1990, 1991) developed PCR-based CE methods for the quantitation of multiple retroviral DNA sequences. An instrument was employed which permitted simultaneous UV and fluorescence (non-LIF) diode-array detection (Schwartz et al., 1989). The sensitivity by UV or (non-LIF) fluorescence was not good enough, however, to allow detection of HIV-1 provirus at very low DNA copy numbers (e.g., for HIV proviral load in asymptomatic individuals). At that time (1991), LIF detection would have provided the extra sensitivity needed but was not yet commercially available. The large increase in sensitivity using an intercalating dye (thiazole orange) and LIF detection (Ar-ion laser) was demonstrated by Schwartz and Ulfelder (1992). Several dsDNA fragments (242, 368, and 900 bp) were detected with ≈ 400 X better sensitivity than possible with UV detection.

Recently, a French research team, Lu et al. (1994), used P/ACE with LIF detection for the quantitative analysis of PCR-amplified HIV-1 DNA or cDNA fragments. The LIFluor dsDNA 1000 Kit (containing the EnhanCE intercalating dye) was used in the CE-LIF experiments. Quantitation of multi-target PCR fragments was demonstrated. Figure 36 shows the CE-LIF electropherogram of three HIV-1 sequences (142 bp, 394 bp, and 442 bp from the gag, pol, and gp41 genes, respectively) together with a DNA standard. Figure 37 shows a dilution series of HIV-1 DNA templates ranging from 1 to 25,000 copies subjected to 40 PCR cycles. A linear range of three orders of magnitude was achieved using CE-LIF. The figure also shows a dilution series obtained from reverse-transcribed (RT) RNA from HIV-1 virions. Data of virion concentrations in sera of individuals infected with HIV-1 at different stages of infection were presented. The measurements by CE-LIF showed excellent correlation with the data acquired with the Southern blot hybridization method.
Figure 36. Detection of multi-target PCR-amplified HIV-1 gag, pol, env sequences by CE-LIF using the LIFluor dsDNA 1000 Kit. A φX 174 RF DNA standard was co-injected with the sample. Reproduced with permission from Lu et al., Nature (London) 268, 269 (1994).

Figure 37. Linearity of LIF-CGE analysis of quantitative PCR or RT-PCR products. Serial dilutions of HIV-1 DNA templates ranging from 1 to 25,000 copies were subjected to a 40-cycle PCR with gag primers SK145/431. RNA extracted from serial dilutions of HIV-1 virions (ranging from 10 to 100,000 viral particles) was reverse-transcribed with 20 pmol of 3’ primer SK431. Reproduced with permission from Lu et al., Nature (London) 368, 269 (1994).
Separations of RT-PCR products from the RNA of polio virus were shown by Rossomando, White, and Ulfelder (1994). Quantitation was achieved by comparing the corrected peak area for the RT-PCR product to a standard curve generated from known amounts of template RNA. The Beckman sieving buffer containing the intercalating dye, EnhanCE, facilitated excellent separation of 53 bp, 71 bp, 97 bp, and 163 bp DNA fragments. The resolution by slab gel electrophoresis, on the other hand, while adequate for the 163 and 97 bp fragments, was inadequate for the other DNA fragments. Figure 38 compares UV (260 nm) vs. LIF detection for a 53 bp RT-PCR product derived from the Sabin 3 strain of virus from the polio vaccine. Note that the migration times of the PCR products are longer for the LIF run as, in this case, the DNA is intercalated with oppositely charged dye. The other important feature to note in Figure 38 is that—in the LIF trace—interferences are far less prominent and the pattern is unambiguous. PCR of target sequences often results in contaminating by-products which can interfere with UV detection.

Figure 38. UV absorbance versus LIF detection of the separation of a 53 bp RT-PCR product from the RNA of the Sabin 3 strain of the polio virus vaccine. A HaeIII-digested φX 174 DNA marker was co-injected with the PCR product for size determination. The same Sabin 3 concentration was used for each analysis. The DNA marker concentration was 200 and 10 µg/mL for the UV and LIF analysis, respectively. Reproduced with permission from Schwartz et al., J. Cap. Elec. 1, 36 (1994). Copyright: ISC Technical Publications, Inc.
5.4.2 Competitive RNA-PCR by CE-LIF for Quantitation of Cellular mRNA

Accurate quantitation of PCR products—especially when dealing with low copy numbers—is often problematic. A group of methods, termed competitive PCR, has been described recently. These methods effectively deal with the problem of accurate quantitation of PCR products (Gilliland et al. 1990; Piatak et al., 1993). In competitive PCR, a known amount of standard template DNA (the “competitor”) competes for the same primers with an unknown amount of target DNA (In the case of RNA-PCR, the DNA is obtained by reverse transcription). The competitor’s sequence is chosen such that it is largely identical to the target sequence, except for the presence of a mutated restriction site or a small intron. During the amplification cycles, the target and competitor are exposed to the same PCR-related reaction variables; their product ratio should, therefore, remain constant, even after the products have reached a plateau. The amount of target DNA (or RNA) can be obtained through a simple interpolation procedure of an experimentally generated standard curve. A variation of this method, termed multiplex competitive PCR, involves co-amplification of the cDNA of a “housekeeping” gene (in addition to amplification of the target and its competitor) whose RNA does not vary among the different samples to be analyzed. The expression of the target gene is then calculated in reference to the housekeeping gene (Apostolakos et al., 1993).

Fasco et al. (1994) recently demonstrated that CE-LIF is an attractive alternative to slab gel techniques. DNA fragments or PCR products, intercalated with the fluorescent dye YOYO-1 (an oxazole yellow dimer) can be detected at extremely low levels in real time with high efficiency and precision. In contrast to the slab-gel-based competitive PCR, the CE method is fast and can be fully automated; the computer-generated data are stored on disk. CE-LIF allows accurate and precise quantitation of PCR products formed during competitive PCR reactions. With the CE method of Fasco et al., excellent peak efficiency was obtained (approximately 10 bp resolution) and run times were less than 30 minutes. PCR product detectability with LIF is adequate for most clinical and diagnostic applications of competitive PCR. The CE-LIF procedure was also applied to multiplex competitive PCR. YOYO-1 was used in the CE run buffer for intercalating the DNA fragments, as discussed in Section 4.3.1.2. The CE-LIF method was applied to reversed-transcribed RNA from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and P4501A1 gene sequences.
A typical competitive RNA-PCR experiment is illustrated in Figure 39. The formation of product (in pmol per 0.1 mL reaction mixture) is plotted vs. the initial RNA concentration. The initial competitor sequence was kept constant at 0.1 amol per 0.1 mL reaction mixture. The inset of Figure 39 shows log-log plot of the ratio of target to competitor product vs. the initial RNA concentration. The intercept at a ratio of 1 corresponds to the initial competitor concentration, i.e., in this case 0.012 µg per 0.1 mL reaction mixture that contains 0.1 amol of target mRNA (assuming 100% reverse transcription efficiency). Representative CE-LIF electropherograms for the competitive RNA-PCR experiments involving P<sub>450</sub>1A1 and GAPDH are shown in Figures 40 A and B, respectively.

Figure 39. Formation of target (■) and competitor (▲) GAPDH during competitive PCR. Equal concentrations were reverse transcribed, mixed at a 1:1 ratio, and diluted. The competitor concentration was kept constant at 0.1 amol/0.1 mL reaction mixture. In the inset plot, the data points obtained at 0.25 and 0.5 µg RNA were omitted. Reproduced with permission from Fasco et al., Anal. Biochem. (1995, in press).
5.4.3 Detection of DNA Polymorphisms and Mutations in Genetic Diseases

Genetic linkage studies follow the inheritance of a particular trait (phenotype) in a family over several generations. The goal is to correlate the trait with the presence of a specific DNA sequence (allele). By finding a marker that is close (“linked”) to the gene, persons predisposed to certain diseases can be identified. Often, years of painstaking work are required to identify the exact location of the gene responsible for the disease on the chromosome. For example, only recently the genes responsible for an inherited form of breast cancer were discovered after 20 years of genetic linkage.
studies (King et al., 1993). Ulfelder et al. (1992) demonstrated the utility of CGE (with UV detection) for restriction fragment length polymorphism (RFLP) analysis of ERBB2 oncogene. This gene was one of the candidates for a breast cancer gene at chromosome 17q and was detected using a PCR method by Hall and King (1991). Polymorphic alleles can be identified by the presence or absence of a specific endonuclease recognition site. RFLPs typically have only two alleles at a given locus. Figure 41 shows the RFLP analysis of three individuals whose genomic, PCR-amplified DNA was digested with MboI. The top two traces represent two homozygous samples characterized by the presence of either the 500 bp or the 520 bp fragment. The heterozygous sample shows both fragments present which were separated in a sieving buffer containing 0.5% HPMC. For comparison, the agarose slab gel of similar samples is also shown in Figure 41.

In a similar study, Del Principe et al. (1993) analyzed PCR-amplified products of the DXS 164 locus in the dystrophin gene. XmnI digestion yielded a polymorphism generating fragments of 740, 520, and 220 bp. The same sieving buffer as that employed by Ulfelder et al. (1992) was used. Another Italian research group (Gelfi et al., 1994 A) investigated an 8 bp deletion linked to congenital adrenal hyperplasia. CE separations were performed with a sieving buffer consisting of 6% linear polyacrylamide. The amplified PCR products were a normal, 135 bp fragment and a disease-linked, 127 bp fragment.

Sensitive LIF detection (P/ACE-LIF combined with a green He-Ne laser) using ethidium bromide (EB) was demonstrated by Liu et al. (1995). The PCR-amplified sequence of the Y-chromosome-specific ZFY gene (307 bp) was detected with much greater sensitivity than possible with EB-stained agarose gel electrophoresis.

DNA microsatellites or short tandem repeats (STRs) are increasingly used as genetic marker systems in linkage studies. They are characterized by tandemly repeated, short (2 to 10 bases) sequences. A tetranucleotide repeat unit (GATT) linked to cystic fibrosis (CF) was studied by Gelfi et al. (1994 B). The allelic forms, a hexamer (111 bp) and a heptamer (115 bp), were amplified by PCR and separated by polyacrylamide gradient slab gel electrophoresis and with CGE (6% linear PA). The hexamer was found linked to the CF-causing mutation in the gene. A sieving buffer consisting of polyacryloylaminoethoxyethanol was used to resolve PCR fragments in the 450 to 550 bp range (Nesi et al., 1994). These fragments were derived from triplet (CAG) repeats in the androgen receptor gene. An increase in the number of triplet repeats is linked to Kennedy’s disease, a neurological disorder.
Figure 41. CE separation of PCR-amplified RFLP samples demonstrating MboI polymorphism. Top, homozygous for allele A1 (520 bp fragment); middle, homozygous for allele A2 (500 bp fragment); bottom, heterozygous for A1 and A2. Constant fragments of 220, 330, and 550 bp can be seen in all three runs as a result of incomplete PvuII digestion of the 550 bp fragment. For comparison, an agarose gel is also shown. Lanes 1, 2, and 8: homozygous (A1). Lanes 3–6: heterozygous (A1 and A2). Lane 10: DNA size markers. Reproduced with permission from Ulfelder et al., Anal. Biochem. 200, 260 (1992).
5.4.3.1 Point Mutations

A number of techniques have been described to detect single-point mutations in DNA (Perucho, 1994). PCR has a central role in the sample preparation step in most of these techniques. Point mutation studies often require that the electrophoretic (or CGE) conditions are chosen such that ds and ssDNA can be separated in one run. In denaturing gradient gel electrophoresis (DGGE), the mobility of a partially melted DNA on the slab gel is reduced compared to an unmelted molecule. A variant of DGGE, termed constant denaturant gel electrophoresis, was recently adapted in a CE format (Khrapko et al., 1994). CGE was performed in capillaries containing a polymer network (6% linear PA) and a denaturant (3.3 M urea and 20% formamide). In a 10 cm portion of the capillary (the “denaturing zone”), the temperature was elevated; in the rest of the capillary, ambient temperature conditions existed. Detection was by LIF. The critical role of temperature is illustrated in Figure 42. Homo- and heteroduplexes from two fluorescein-labeled DNA fragments (206 bp) originating from human mitochondria were resolvable by tuning the temperature. The two homoduplexes differed by a single bp substitution (GC vs. AT). At 31°C, a single peak was obtained, indicating that all the duplexes were in the unmelted form. As the temperature was raised, the duplexes started to separate in the order of their melting stability. At 40°C, all the species eluted again in one single peak as their partially melted duplexes. The sensitivity of the CGE method is such that 10^5 mutant species can be detected among 3 × 10^8 wild-type sequences.

A similar type of point mutation method, called heteroduplex polymorphism analysis (HPA), was proposed by Cheng et al. (1994). Duplexes and ssDNA were separated in a polymer network consisting of 0.5% HPMC and 4.8% glycerol. Ethidium bromide (3 µM) was added to increase resolution (see Section 4.2.4). The sensitivity of the CE method was not as sensitive as the one discussed above, as UV detection instead of LIF was used. However, the authors contend that their detection system does not require the provision of natural or artificial GC-clamp domain. The latter are required to provide duplex stability in heteroduplex molecules under denaturing conditions.
Figure 42. Constant denaturant CGE separation as a function of column temperature. The sample, an equimolar mixture of two homoduplexes (GC and AT) and two heteroduplexes (GT and AC), was prepared using fluorescein-labeled DNA fragments and run at the several temperatures indicated. Reproduced with permission from Khrapko et al., Nucl. Acids Res. 22, 364 (1994).

Another technique for the screening of point mutations is single-strand conformation polymorphism analysis (SSCP). This technique, originally developed by Orita et al. (1989), takes advantage of differences in mobilities between DNA fragments in non-denaturing gels. Point mutations in the DNA will cause conformational changes resulting in the mobility differences of ssDNA (Guttman et al., 1992 A).

Kuypers et al. (1993) studied the p53 gene located on the short arm of chromosome 17. CGE was run on control and patient (multiple myeloma) samples, using a 4% linear PA polymer network in the capillary. Denatured samples of normal DNA showed two peaks corresponding to the two ss species. The control cell line and patient samples revealed more complicated patterns of 3 to 5 peaks. Fraction collection by CGE (Section 4.4) was used to confirm the presence of different sequences in these peaks. A recent paper from the same group (Kuypers et al., 1994) dealt with quanti-
tation of residual lymphoma cells carrying a translocation between chromosomes 14 and 18 in patient blood samples that were amplified by competitive PCR.

In the amplification refractory mutation system (ARMS) or allele-specific amplification (ASA), PCR is used to detect point mutations without requiring endonuclease digestion or Southern hybridization. The utility of CE-LIF to detect mutations in the phenylketonuria gene by the ARMS method was demonstrated by Arakawa et al. (1994 A).

Another paper by the same group (Arakawa et al., 1994 B) describes the utility of the CE-LIF method for diagnosis of medium-chain coenzyme A dehydrogenase (MCAD) deficiency, a disorder linked to sudden infant death and Rye-like syndromes. In most cases (90% of mutant alleles) the MCAD deficiency is caused by a single, A-to-G nucleotide change at position 985 in the gene. DNA fragments were amplified by two sets of allele-specific oligonucleotide primers. Mutant alleles yielded a single, 175 bp fragment, normal alleles yielded a 202 bp fragment, whereas heterozygous carriers produced both fragments. The DNA fragments were well resolved within a 12 minute run time on a capillary filled with low crosslinked PA. The CE-LIF method was linear over 3 orders of magnitude with a detection limit of $\approx 10$ ng/mL for a 603 bp DNA fragment. Compared to UV detection, LIF was 100 times more sensitive.

### 5.4.4 DNA Profiling in Forensic Work

CE-LIF has recently been applied in the analysis of genetic markers for human identification. Because often extremely low levels of substances are investigated, LIF should be the detection method of choice. Therefore, several researchers have studied different polymer matrix-fluorescent dye systems to optimize separation efficiency and detectability. Variable number tandem repeat (VNTR) analysis of the amplified D1S80 locus (300 to 700 bp) with P/ACE-LIF is shown in Figure 43. D1S80 has a repeat unit of 16 bp. The EnhanCE dye was used in the polymer-network-containing run buffer. The figure shows the alleles for homozygous and heterozygous individuals.
Srinivasan et al. (1993) compared two asymmetric cyanine dyes, TOTO-1 and YOYO-1 (Molecular Probes, Eugene, OR), with the 488 nm Ar-ion laser. Three genetic marker systems (apolipoprotein B, 700–1000 bp with a 14 bp repeat; VNTR locus D1S80, 300 to 700 bp with a 16 bp repeat; and mitochondrial DNA, 130 to 140 bp with a 2 bp repeat), were investigated for forensic applicability by PCR amplification. The PCR products were subsequently pre-stained with the fluorescent dye (DNA was added to dye at a molar ratio of 5:1 DNA bp to dye and incubated for 20 minutes prior to analysis). Capillaries containing easy-to-use, replaceable, polymer network solutions (0.5% methylcellulose) were found superior to crosslinked PA gels (3%C, 3%T).

McCord et al. (1993) analyzed some other genetic marker systems of forensic interests, i.e., the human myelin-basic protein gene, the von Willenbrand Factor gene, and the HUMTH01 gene located on chromosome 11. PCR-amplified alleles resulting from VNTRs with 4 bp repeat units in the 100 to 250 bp range were separated with replaceable, polymer network solutions. An asymmetrical dye, YO-PRO-1 (Molecular Probes, Eugene, OR) was added to the polymer solution of 1.0% hydroxy-

---

**Figure 43.** VNTR analysis of the amplified D1S80 locus for homozygous and heterozygous individuals using LIFluor dsDNA 1000 Kit and CE-LIF detection. Courtesy of K. J. Ulfelder, Beckman Instruments, Inc.
ethylcellulose and to each DNA sample for CE-LIF detection. Later studies from this group (Butler et al., 1994 A and B) focused on quantitative aspects of the CE-LIF method for forensic DNA typing. Comparisons with other existing methods (slab gel, slot blot, fluorescence spectrophotometry) were also made. With an internal standard, peak migration time precision was < 0.1% RSD. Peak area precision—using pressure injection—was ≈ 3% RSD (see also Section 4.2.7, Table 4). A single-step voltage gradient allowed shorter run times for the HUMTH01 allelic ladder analysis (< 10 min) while still maintaining ≈ 3 bp resolution in the region of interest (Butler et al., 1994 B).

5.4.5 DNA Profiling of Plants, Bacteria and Fungi

Identification of bacteria in clinical samples using standard culturing techniques is both time consuming and cumbersome since the bacteria must be grown in the laboratory and identified on the basis of nutritional development requirements. In addition, many bacteria are morphologically similar to one another. Avaniss-Aghajani et al. (1994) have developed a method for the identification of various bacterial species using PCR amplification of small subunit ribosomal RNA genes, which vary in sequence among bacterial species. PCR was accomplished using one set of primers, one of which was 5'-labeled with fluorescein isothiocyanate (FITC). Subsequent fluorescently labeled PCR products were then subjected to digestion with restriction endonucleases, producing fragments of different length due to variations in sequence among the bacterial species. When analyzed by CE-LIF (Ar-ion laser at 488 nm), only the DNA fragments containing the terminal 5'-FITC label were detected. The length of the labeled restriction fragment was then used to identify a particular bacterial species. Figure 44 shows the CGE analysis of PCR products for four bacterial species (Escherichia coli, Flavobacterium okeanokoites, Klebsiella pneumoneae, and Streptococcus faecalis) after digestion with endonucleases MspI and RsaI. Use of this process will result in clear bacterial identification with major time savings.

Size-selective DNA profiling and RFLP analysis of amplified polymorphic spacers originating from fungus rDNA was performed by Martin et al. (1993) using the CGE conditions of Schwartz et al. (1991). Inter- and intraspecific variation in the size and number of restriction sites of the amplified rDNA spacers from several fungi were examined, allowing the strains to be genotyped by CGE (UV detection).
Figure 44. CE-LIF electropherogram of PCR-amplified SSU rRNA genes of Flavobacterium okeanokoites, Escherichia coli, Streptococcus faecalis, and Klebsiella pneumoneae after digestion with MspI and RsaI. Numbered peaks correspond to the 5' terminal restriction fragments of the digested PCR products. Peak 1: MspI digest from F. okeanokoites. Peak 2: RsaI digest from E. coli. Peak 3: MspI digest from S. faecalis. Peak 4: MspI digest from K. pneumoneae. Electropherogram courtesy of E. Avaniss-Aghajani, UCLA.

Marino et al. (1994) showed results of soybean genotyping using CGE with crosslinked gels. Polymorphism was detected in dinucleotide, short tandem repeat sequences (STRs). Figure 45 shows the allelic STR profiles of two genotypes (Williams and Jackson), together with the F$_1$ progeny. The latter is heterozygous, containing both of the parent alleles. In the electropherogram, the alleles are located between the molecular mass markers of 100 bp (29 minutes) and 200 bp (36 minutes).
Figure 45. Analysis of the parental genotypes Jackson and Williams and their F₁ progeny. The molecular mass markers 100 and 200 base pairs are at 29 and 36 minutes, respectively. The SSR-containing fragments for the genotypes are found between 30 and 33 minutes. F₁ generation is heterozygous. Reproduced with permission from Marino et al., J. Chromatogr. A 676, 185 (1994).

5.4.6 Plasmid Mapping

Restriction enzyme digestion of plasmids (“plasmid mapping”) is often used for confirmation of PCR products, in cloning experiments, and in biotechnology process control, e.g., to monitor genetic stability. Maschke et al. (1993) employed CGE (UV detection) with replaceable, 6% linear PA gels for the mapping of four closely related plasmids. Various high-resolution plasmid maps were shown, obtained with a number of different restriction enzymes. The number of theoretical plates (based on a 242 bp fragment) was calculated as ≈ 3 million per m. In particular, the smaller DNA fragments are better resolved by CGE than with agarose gel electrophoresis. Compared to the slab gels, CGE for plasmid mapping applications is quantitative, fast (run times < 20 min), and consumes minimal amounts of sample for analysis. Comparisons between CGE (6% linear PA) and agarose slab gels for plasmid restriction digests were also pub-
lished by Paulus and Husken (1993). In the analysis of plasmids, the polymer network concentration and/or chain length must be optimized to accommodate the larger sizes of the DNA fragments to be separated (see Section 5.5, below).

### 5.5 dsDNA (2 to 20 kbp) by CGE

As in slab gel electrophoresis, the size range to be separated in CGE can be extended by diluting the polymer concentration of the sieving matrix. Some examples of this were shown earlier for alkylcellulose polymer solutions (Figures 17 and 18). With linear-PA-based gels, a 3% polymer network was found suitable to separate efficiently a 1 kbp DNA ladder (range: 72 to 12216 bp), with some peaks exhibiting in excess of 4 million plates per m (Pariat et al., 1993). Similar capillary separations were obtained by Strege and Lagu (1991) and Baba et al. (1993) on alkylcellulose polymer networks, by Bocek and Chrambach (1992) on 2% SeaPrep agarose, and by Chiari et al. (1994) on a polyacryloylaminoethoxyethanol replaceable gel. Pariat et al. (1994) showed that low concentrations of linear PA (1.5%) can be used to further extend the size range, e.g., for the separation of λDNA HindIII restriction fragments. This digest contains fragments ranging from 125 bp to 23.1 kbp (Figure 46). Others (Barron et al., 1994; Kim and Morris, 1994 A) have recently demonstrated that ultradilute solutions (∼0.01%) of alkylcellulose also can be used for these type of separations.

---

**Figure 46.** Electropherogram of λHind III DNA using 1.5% linear polyacrylamide. Peak identification: 1 = 564 bp; 2 = 2.0 kbp; 3 = 2.3 kbp; 4 = 4.4 kbp; 5 = 6.6 kbp; 6 = 9.4 kbp; 7 = 23.1 kbp. Reproduced with permission from Pariat et al., J. Chromatogr. A 652, 57 (1993).
5.5.1 Quantitation of Plasmid Copy Number

In recombinant DNA technology, plasmid analysis is often used to control the genetic stability during fed-batch culture. Generally, the size range for plasmid analysis is larger than for PCR product analysis, i.e., 2000 to 22,000 bp. *E. coli* is the most frequently used host cell in the production of recombinant molecules. However, host cell-plasmid systems have limited genetic stability. During cell cultivation, therefore, the plasmid concentration (copy number) may decrease. Hebenbrock *et al.* (1993) have shown that CGE is an excellent quantitative tool to monitor the plasmid concentration during the cultivation of the *E. coli* strain containing the plasmid. The plasmid DNA concentration was estimated from the integrated peak areas of an internal standard (4,363 bp) and the plasmid carrying the genetic information (a linearized, 13,000 bp restriction fragment). The CGE method used 4% linear PA polymer networks for separation of DNA fragments ranging from 3,000 to 22,000 bp.

5.6 Very Large Chromosomal DNA (> 20 kbp)

Preliminary results of separations of very large DNA fragments were shown by Chrambach’s group (Guszczynski *et al.*, 1993). Linear-PA-filled capillaries (0.1 to 0.9%) were used for three DNA-containing samples in the size range of 20 to 50 kbp, multiples of 50 kbp, and 3 to 6 Mb, respectively. However, as noted by the authors, various problems related to the sampling and the CE instrumentation render their CGE method as yet impractical for large DNA. Pulsed-field CE, in conjunction with dilute polymer solutions (see Section 3.2.5), may, in fact, be a more promising approach as recent publications indicate (Sudor and Novotny, 1994; Kim and Morris, 1994).
6 References


Bae, Y. C., Soane, D. *J. Chromatogr.* 652, 17 (1993)

Banke, N., Hansen, K., Diers, I. *J. Chromatogr.* 559, 325 (1991)


Bocek, P., Chrambach, A. *Electrophoresis* 13, 31 (1992)


Chargaff, E., Saidel, H. F. *J. Biol. Chem.* 177, 417 (1949)


Chiari, M., Nesi, M., Righetti, P. G. *Electrophoresis* 15, 616 (1994)


Compton, S. W., Brownlee, R. G. *BioTechniques* 6, 432 (1988)


Edstrom, J. E. *Nature* 172, 908 (1953)


Foret, F., Szoko, E., Karger, B. L. *J. Chromatogr. 608*, 3 (1992)


Guttman, A., Nelson, R. J., Cooke, N. *J. Chromatogr. 593*, 297 (1992 A)


Kasai, K. J. *Chromatogr.* 618, 203 (1993)


Lerman, S. L and Frisch, H. L.  *Biopolymers* 21, 995 (1982)


Maschke, H. E., Frenz, J., Belenkii, A., Karger, B. L., Hancock, W.  *Electrophoresis* 14, 509 (1993)


Ng, M., Blaschke, T. F., Arias, A. A., Zare, R. N. *Anal. Chem.* 64, 1682 (1992)


Oefner, P. J., Bonn, G. K. *Am. Lab.* 26, 28C (1994)


Piatak, M., Jr., Luk, K.-C., Williams, B., Lifson, J. D. *BioTechniques* 14, 70 (1993)


Southern, E. M. *J. Mol. Biol.* 98, 503 (1975)


Volkmuth, W. D., Austin, R. H. *Nature* 358, 600 (1992)


