Capillary Electrophoresis of DNA

DNA fragments are traditionally separated and analyzed by slab gel electrophoresis. The gel matrices are usually either polyacrylamide (*UNIT 10.4*) or agarose, and separations are achieved in the presence (for ssDNA) or the absence (for dsDNA) of dissociating agents such as urea or formamide. The slab gel systems have the advantage of analyzing multiple samples in the same separation at low cost, but normally take several hours to complete. The DNA is typically visualized with stains, UV shadowing, intercalating dyes such as ethidium bromide, and on occasion by radioactivity. Capillary electrophoresis (CE), an alternative to conventional slab gel electrophoresis, has developed over the past few years into a very powerful tool for the separation of DNA fragments. CE offers a number of advantages over slab gel separations in terms of speed, resolution, sensitivity, and data handling. This is partly because the CE separation occurs inside a small-diameter (50- to 100-μm) quartz capillary in the presence of high (kilovolt-level) separating voltages. Separation times are generally only a few minutes. The DNA is detected either by UV absorption or by fluorescent labeling, both of which eliminate the need to use mutagenic substances (e.g., ethidium bromide) or dispose of radioactive waste. The quantity of DNA required for the separation is in the nanogram range. Single-base resolution can be readily obtained on fragments up to several hundred base pairs in size. In the presence of appropriate standards, fragments can be accurately sized, based on relative electrophoretic mobility.

The separation of DNA fragments by CE occurs within the walls of a fused-silica capillary. Since the negatively charged nature of this surface has a dramatic impact on the resolution achieved during the separations, the vast majority of CE separations are done in "coated" capillaries whose surface has been modified to be chemically inert to the DNA. The capillaries are filled with a sieving matrix, and the DNA fragments are separated on the basis of size, analogously to standard slab gel separations. The matrix is either a chemically cross-linked gel, such as polyacrylamide, or a flowable polymer, such as modified cellulose or non-cross-linked polyacrylamide. Single-stranded DNA (ssDNA) fragments as small as 5 bases are readily separated with single-base resolution. The analysis of synthetic oligonucleotides in a flowable matrix is described in this unit (see Basic Protocol 1) as an example of this type of application. Fragments of double-stranded DNA (dsDNA) as large as 20 kb are also separated, although not with single-base-pair resolution. The only difference between these separations is the separation matrix.

CE has found increasing use in a number of analytical applications where DNA separations are required. These include assessment of the purity of synthetic oligonucleotides and their modifications, analysis of PCR products, sequencing of fluorescent DNA, analysis of restriction maps, accurate sizing of restriction fragments for genetic analysis, forensic analysis of biological samples, genotyping, and analysis of conformational polymorphisms. Additional applications continue to be developed. An area of growing interest is the ability to analyze low levels of PCR products in biological fluids, as presented below (see Basic Protocol 2). Rapid progress is also being made in the development of multicapillary automated DNA sequencing instruments using laser fluorescence detection.

CE is an analytical technique rarely used in preparative mode. This is largely because only small quantities of DNA can be loaded onto a capillary. Amplifying DNA by PCR after separation can circumvent this problem. In general, however, preparative separation of DNA fragments is best achieved by slab gel electrophoresis (*UNIT 10.4*) or high-performance liquid chromatography (HPLC) methods (*UNIT 10.5*).

Contributed by Alan Smith and Robert J. Nelson
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INSTRUMENTATION

CE separation in its simplest form can be achieved by passing a high voltage between two buffer reservoirs that are joined by a fused silica capillary filled with liquid or gel. This results in an electric field that drives the molecules of interest from one end of the capillary to the other. The capillaries are generally 20 to 80 cm long and 50 to 100 µm in internal diameter, with total volumes in the 1- to 2-µl range. For comparison, the volume of a slab gel lane is ~1000 µl. The capillaries are thin walled, which allows for dissipation of the Joule heating resulting from the high voltages (10 to 30 kV) that are necessary for high-performance electrophoretic separations. This minimizes convective effects that could result in band broadening during electrophoresis. The fused-silica capillary is coated on the outside with a polyimide layer that eliminates oxidation of the fused-silica glass and confers excellent tensile strength to the otherwise fragile capillary. The polyimide sheathing is carefully burned from a small portion of the capillary to expose a section of the silica. This clear section of the capillary is inserted into the light path of a UV or fluorescence detector and becomes the on-column flow cell. As the DNA molecules migrate through the capillary as a result of the electric field, they pass through the detector light path and are measured by UV or fluorescence detection. In effect, the separation column itself becomes a very-low-volume flow cell.

IMPORTANT NOTE: Removal of the polyimide coating makes the capillaries susceptible to breakage. Capillaries that are not provided in cartridges by the manufacturer should be handled with care to avoid breakage.

The combination of high field strength and large surface-area-to-volume ratio of the capillaries results in rapid and very efficient separations of both ssDNA and dsDNA. Sample loading can be accomplished from as little as 1 µl, with starting sample concentrations of ~1 µg/ml for UV detection and ~1 pg/ml or less for laser-induced fluorescence detection. Clearly, with respect to sensitivity, speed, and versatility, CE offers significant advantages over gel electrophoresis for the separation of nucleic acids.

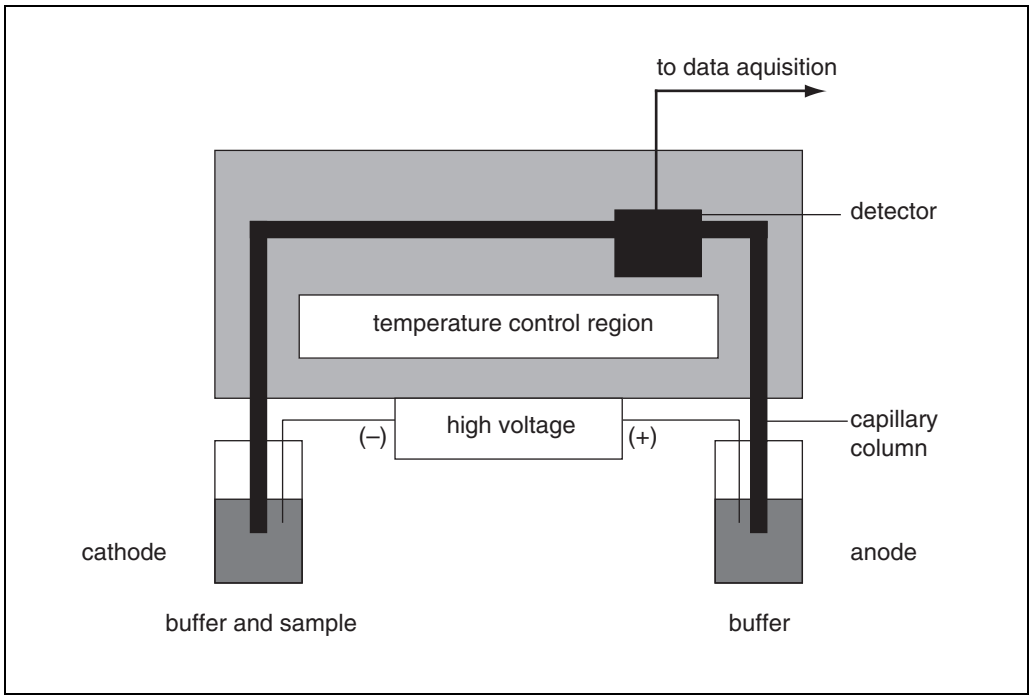

Figure 10.9.1 Schematic of a CE instrument configured for DNA separations.

Table 10.9.1 Capillary Electrophoresis Systems

Manufacturer	Model number[a]	Autosampler	Detection method[b]	Column temperature
Agilent	CE System	48-position, 10° to 40°C	Diode array UV-vis	15° to 60°C
Amersham	MegaBACE 500 16, 32 or 48-capillary DNA sequencer	96-well plate, no cooling	4-color scanning LIF detection	27° to 44°C
	MegaBACE 1000 96-capillary DNA sequencer	Single 96-well plate, no cooling	4-color scanning LIF detection	27° to 44°C
	MegaBACE 4000 384-capillary DNA sequencer	Single 384-well plate, no cooling	4-color scanning LIF detection	27° to 44°C
Applied Biosystems	Prism 310	482- or 96-sample position, cooled 96-well	4-color LIF with CCD	30° to 60°C
	Prism 3100 16-capillary DNA sequencer	96-well plate with sample cooling	CCD	26° to 65°C
	Prism 3730 96-capillary DNA sequencer	96-well plates with autoloader	CCD	18° to 70°C
Beckman Coulter	P/ACE MDQ DNA System	Up to 96-well plate	UV and diode array detection	15° to 60°C
	CEQ 8000 8-capillary DNA sequencer	96-well plate with cooling and heating to 90°C	4-color CCD	30° to 60°C

[a]All units listed have a single capillary unless otherwise noted.

[b]LIF, laser-induced fluorescence detector, used with either a PMT (photomultiplier tube) or a CCD (charge-coupled device) camera for visualization; UV, ultraviolet detector.

As stated previously, in its simplest form capillary electrophoretic separation can be achieved by passing a current between cathodic and anodic buffer reservoirs via a liquid-filled glass capillary. In practice, the basic CE instrument also requires a suitable sample injection module, a detector, adequate temperature control, and isolation for the user from the high voltages used for the separations. A schematic of the basic instrument is shown in Figure 10.9.1. There have been a number of changes recently in the features of commercially available instruments; these instruments and their capabilities are summarized in Table 10.9.1.

SEPARATION THEORY

CE is part of the family of electrophoretic techniques that separate species based upon their size and ionic properties. An ion (i) placed in an electric field will move in the direction parallel to the field towards the oppositely charged electrode with a velocity (v_i) defined as follows:

$$v_i = \mu_i E = \mu_i V/L$$

where μ_i is the mobility of the ion, E is the electric field in volts per centimeter, V is the voltage across the column, and L is the total column length. The electrophoretic mobility of a given ion is equal to:

$$m_i = q_i/6\pi\eta a_i$$

where q_i is the charge on the ion, η is the viscosity of the buffer or gel matrix, and a_i is the radius of the ion.

Since DNA has a constant size-to-charge ratio, a sieving matrix must be added to the capillary in order to discriminate based only on size, rather than charge and size. In CE there are two types of gels employed in DNA separations: cross-linked gels (static gels) and non-cross-linked gels (flowable polymers or polymer networks). Cross-linked gels are fixed gels that are polymerized inside the capillary, usually covalently bound to the capillary surface, and are not removed from the capillary between runs. Flowable polymers are viscous hydrophilic polymer solutions that can be pumped into the capillary. The same flowable polymer matrix can be used repeatedly when small molecules such as synthetic oligonucleotides are being analyzed. The time between injections is sufficient for the preceding sample to clear the detector. Alternatively, the polymer can be used once, discarded, and replaced with fresh matrix prior to the next injection. This approach is preferred where larger DNA molecules are present in the samples—e.g., for fragment analysis and DNA sequencing analysis. Usually, a coated capillary is utilized to eliminate the charge effects that are contributed by the native silica surface. With cellulose-derived polymers or some specially modified acrylamides, however, uncoated capillaries may be used, because of the strong interaction of the polymer with the inner surface of the bare fused-silica capillary, in essence forming its own coating.

With either a cross-linked or non-cross-linked gel in the capillary, the matrix offers a frictional resistance to the movement of the DNA through the gel medium that is proportional to the size of the species. The frictional resistance can vary with the molecular weight, concentration, and chemical composition of the flowable gel polymer or the pore size in the cross-linked gel, and must be optimized for the particular size of the DNA to be separated. A detailed description of the theory of DNA motility in entangled polymer solutions can be found in Grossman (1991).

STRATEGIC PLANNING

The most common approach to the separation of both ssDNA and dsDNA by CE uses a coated capillary and an uncharged sieving matrix. This is very similar to slab gel electrophoresis, but in a silica capillary. The separation matrix, as mentioned, can take the form of a cross-linked polyacrylamide gel or flowable polymer such as hydroxypropyl methyl cellulose (HPMC), hydroxyethylcellulose (HEC), polyethylene oxide (PEO), or non-cross-linked linear polyacrylamide. The cross-linked gel is polymerized directly inside the capillary and can be reused for 30 to 100 separations before losing resolution. The capillary is then discarded, since the polyacrylamide gel cannot be regenerated. The flowable polymer has the advantage that it can be expelled from the capillary by pressure at the end of each electrophoretic separation; fresh matrix is then reloaded into the capillary prior to the next separation. These capillaries have lifetimes of several hundred injections. The eventual loss of the surface coating is the major reason for replacement; another common reason is mechanical breakage.

The selection of the appropriate matrix can significantly affect the quality of the separation. Cross-linked polyacrylamide is best used for the separation of synthetic oligonucleotides—both native and modified versions. However, flowable polymers can also be used for oligonucleotide analysis and for the separations of automated sequencing ladders. Where dsDNA fragment analysis is required, only flowable polymers are routinely used. The general rule for matrix selection is that the larger the DNA fragment, the weaker the sieving capabilities of the matrix.

Separation buffers frequently are variants of Tris/borate/EDTA (TBE) mixtures and are buffered at alkaline pH. Urea is often included in the buffer, as a denaturant, when analyzing ssDNA (e.g., synthetic oligonucleotides). Samples are loaded onto the capillary by electrokinetic, or pressure, injection. Separation times range from 10 to 45 min, at

voltages between 1 and 10 kV. DNA fragments are detected in the UV spectrum at 260 nm, either in the presence or absence of ethidium bromide. Sensitivity can be increased by at least two orders of magnitude through the use of fluorescence detection. Since DNA possesses no native fluorescence, intercalating dyes such as cyanine derivatives (Zhu et al., 1994) or rhodamine derivatives must be added to the electrophoresis buffer, or covalently attached to the DNA prior to the electrophoretic separation. In addition to increasing the sensitivity of detection, these intercalating dyes can improve resolution and sharpening of the bands by physically disrupting the DNA structure. The selection of specific dyes is dictated by their excitation and emission spectra and the compatibility with the detection systems of individual instruments. Specific examples of intercalating dyes are: thiazole orange (Aldrich), YO-PRO-1, YOYO-1, and Sybr Green (Molecular Probes). The added sensitivity is particularly useful when analyzing PCR products that have been amplified from biological fluids. Targets of interest are frequently present in small amounts, and the presence of salts and proteins make their direct analysis by CE impractical. However, after completion of the PCR reaction, the sample can be diluted with water and an aliquot analyzed using fluorescent detection.

The mobility of a given DNA fragment may not be constant over a series of injections. This variability can have a variety of causes: aging of the polymer (polyacrylamide), loss of capillary coating, or depletion of the conductivity of the running buffers. The absolute mobility of DNA in a given sample will be dependent upon the salt content (and hence the conductance) of that sample. The presence of high salt will significantly reduce the electrophoretic mobility of the DNA. One solution is to dilute the sample in water and load for longer times; alternatively, the sample can be desalted (*UNIT 10.7*) prior to injection. Where accurate sizing is important, it is essential to incorporate sizing standards into the sample prior to electrophoresis.

The CE analysis of synthetic oligonucleotides requires the selection of a matrix that optimizes resolution of low-molecular-weight oligonucleotides. The separation of fluorescently labeled fragments from an automated sequencing ladder represents a specialized CE application and requires the selection of a matrix with a greater resolution range. These ladders range in size from 20 to more than 1000 bases and can be separated with single-base resolution to a high degree of accuracy. Currently, three automated CE instruments are commercially available as DNA sequencers (see Table 10.9.1). A detailed discussion of the general principles associated with dideoxy sequencing can be found in Ausubel et al. (2003).

SEPARATION OF OLIGONUCLEOTIDES

BASIC PROTOCOL 1

In this protocol synthetic oligonucleotides are analyzed for purity by CE using a replaceable, flowable polymer as the separation matrix. The running buffer and separation matrix contain 7 M urea to keep the DNA in its single-stranded configuration. The sample is loaded onto the capillary at the cathode by electrochemical injection. After loading, the sample vial is replaced by the cathode buffer reservoir, and the electrophoresis is continued. The matrix does not need to be replaced between each separation, but should be replaced at the beginning of each series of separations, e.g., at the beginning of each day. Each time fresh matrix is loaded, the capillary must be equilibrated before samples are run. No further equilibration is required between samples. The electrophoretic separation should provide single-base resolution for DNAs of at least 100 bases. A poly$(A)_{40\text{-}60}$ size ladder (see Fig. 10.9.2) should be analyzed initially in order to confirm that resolution is optimal.

NOTE: The filled capillary can be stored on the instrument overnight, but if >1 day elapses between runs, the capillary should be stored at 4°C with both ends capped.

NOTE: The following protocol demonstrates the use of a P/ACE 5510 CE instrument from Beckman Coulter. However, other instruments (Table 10.9.1) are capable of comparable separations when operated in accordance with manufacturers' instructions.

NOTE: The selection of a matrix is often instrument-dependent. It is recommended that a kit be used initially for a reference separation. Linear polyacrylamide is frequently used for oligonucleotide separations.

Materials

ssDNA 100-R separation kit (Beckman Coulter) including:
60-cm, 100-µm-i.d. coated capillary
ssDNA 100-R separation gel solution
Running buffer: Tris-borate electrophoresis buffer (reconstitute and store up to 30 days at 4°C)
Poly(A)$_{40\text{-}60}$ sizing standard (dissolve at 100 µg/ml [3 OD_{260} units/ml] in water and store indefinitely at −20°C)
Dried ssDNA oligonucleotide sample
CE instrument (e.g., Beckman Coulter P/ACE 5510 or equivalent; see Table 10.9.1)

1. Reverse standard polarity of the CE instrument electrodes (see manufacturer's instructions).
2. Rinse capillary on-instrument with deionized water for 5 min.
3. Fill the capillary with ssDNA 100-R gel solution using a 20-min pressure rinse from the matrix vial (based on a 20-psi rinse pressure).

This solution can be stored on-instrument for 5 days, but should then be discarded.

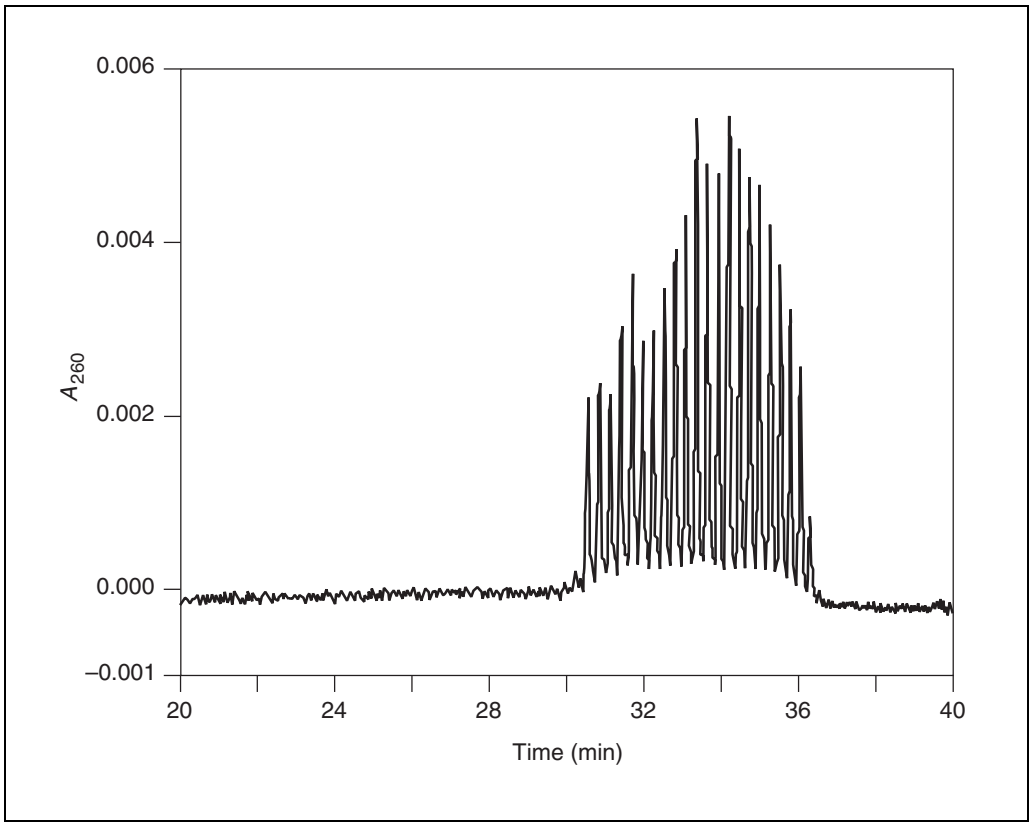

Figure 10.9.2 CE separation of a standard poly(A)$_{40\text{-}60}$ mixture of synthetic oligonucleotides.

4. Equilibrate the capillary in running buffer by running a voltage ramp from 0 to 8.1 kV over 20 min and holding at 8.1 kV for 10 min.
5. Replace the inlet reservoir with a container of water and inject for 1 sec at 7.5 kV.
6. Position the sizing standard vial at the inlet and inject for 10 sec at 7.5 kV.
7. Replace with running buffer reservoir and carry out the electrophoresis at 8.1 kV for 40 min at 30°C.
8. At completion of run, confirm that the separation of the standards is satisfactory by comparison with the example provided in the kit.
9. Prepare samples to be analyzed by dissolving in water to ~10 µg/ml.
10. Load samples onto autosampler and inject for 10 sec at 7.5 kV.
11. Carry out the electrophoresis at 8.1 kV for 40 min at 30°C.
12. Repeat steps 10 and 11 until resolution begins to deteriorate (at least 15 runs); then replace separation gel by returning to step 2.

QUANTITATIVE PCR ANALYSIS

BASIC PROTOCOL 2

Quantitative PCR can be used in conjunction with CE separation to amplify and quantitate any DNA target sequence—by the use of either an intercalating dye (coinjected with the samples) or a covalently modified, fluorescently labeled oligonucleotide primer. The size of the expected product is determined by coinjection of sizing standards. Quantitation is achieved by the coamplification of a second target sequence of known concentration, or by the addition of a known quantity of DNA to each sample.

Another application of this method is the direct measurement of viral load by reverse transcription (RT)-PCR of the viral RNA. This is achieved by the procedure known as competitive PCR analysis (Piatak, 1993). A known amount of a standard DNA template is included in the reaction mixture to compete for amplification with the target DNA. The sequence of the competing DNA is designed such that the PCR product is similar, but not identical, in size to the target DNA. The small quantities of DNA that are produced by this process require coinjection of an intercalating dye (e.g., YO-PRO-1 or Sybr Green I) as well as fluorescence detection.

Since dsDNA is being analyzed in these applications, it is not necessary to include denaturant in the electrophoresis buffer.

Materials

LIFluor dsDNA 1000 kit (Beckman Coulter) containing:
- Gel buffer mixture (containing separating gel and Tris/borate/EDTA buffer)
- EnhanCE intercalating dye
- 65-cm, 100-µm-i.d. coated capillary
- Standard sizing ladder: *Hae*III restriction digest of ϕX-174 DNA (dissolve at 10 µg/ml in deionized water and store at −20°C)

PCR reaction mixes containing amplicon

CE instrument with fluorescent detection (e.g., Beckman Coulter P/ACE 5510 or equivalent)

1. Prepare gel buffer mixture according to manufacturer's instructions and add 0.4 µg/ml intercalating dye.

   Store up to 30 days at 4°C.

2. Reverse standard polarity of CE instrument electrodes (see manufacturer's instructions).

3. If the capillary is new, rinse with gel buffer for 10 min at high (20 psi) pressure.

   This step is not necessary between runs.

4. Place standard sizing ladder in inlet position and load for 10 sec at low (0.5 psi) pressure.

   See Figure 10.9.3 for a chromatogram of this standard.

   The buffer ions become depleted over a series of injections. Consequently the inlet gel reservoir should be replaced after 30 injections.

5. Perform electrophoresis at 9.4 kV for 30 min at 25°C.
6. At the completion of the separation, replace the gel matrix using a 3-min high-pressure wash.
7. Assess the resolution and the linearity of area quantitation of the electrophoretic separation, and compare with the expected profile.
8. Dilute the PCR reaction mixes 10-fold with water and analyze by repeating steps 4 to 6, as with the sizing ladder.

   Some applications may require the coinjection of the sizing ladder with the sample. In these instances the sizing ladder is loaded for 10 sec, followed by the sample for 10 sec.

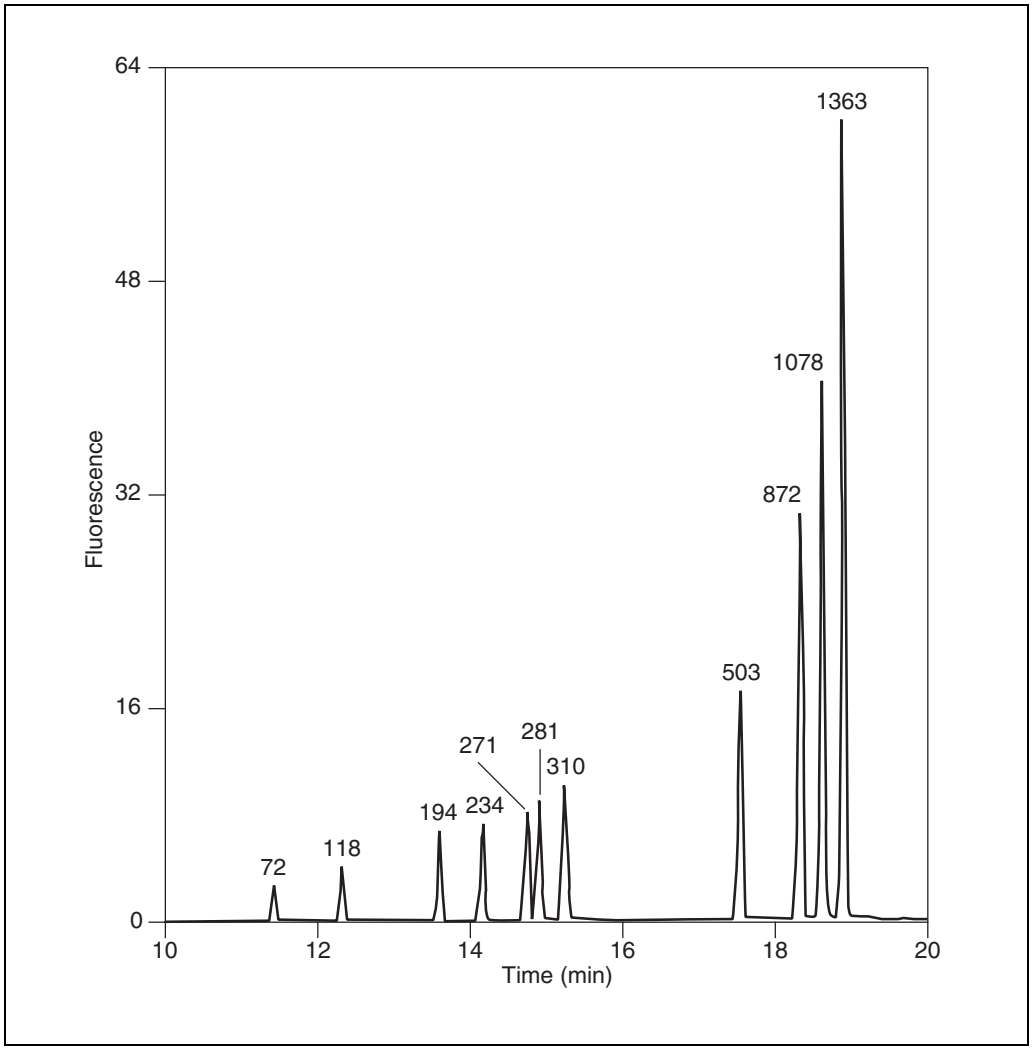

Figure 10.9.3 A sizing ladder from a *Hae*III digest of ϕX-174 DNA. Fragment size in bp is indicated.

COMMENTARY

Background Information

The application of capillary electrophoresis to the separation of DNA fragments is a relatively recent development. The inherent advantages of this technology—high resolution, excellent sensitivity, and rapid separation times—provide significant improvements over the conventional slab gel electrophoresis technology. This has led to rapid acceptance of CE as an essential tool for the analysis of DNA fragments of all sizes.

When applied to the analysis of synthetic oligonucleotides (see Basic Protocol 1), the technique's ability to obtain single-base resolution has proven extremely useful in diagnosing synthesis problems and in obtaining overall purity estimates for the final products (Cohen et al., 1988). The chemistry of DNA synthesis involves sequential addition of bases to the growing oligonucleotide chain in a prescribed order (*APPENDIX 3C*). After each addition, the growing chain is "capped" in order to terminate the portion of the oligonucleotide that has not completed the coupling reaction. This yields a truncation series. If present in sufficient quantity, these species will compete with the full-length oligonucleotide in some applications. Since they are shorter than the full-length oligonucleotide they can readily be resolved and quantitated by CE.

After synthesis has been completed, the bases have to be deprotected. If deprotection is incomplete it can interfere with the base-pairing properties of the oligonucleotide. These species, which appear larger than the full-length oligonucleotide, can also be readily resolved by CE. Figure 10.9.4 illustrates a crude oligonucleotide containing coupling failures (shorter migration times) and incompletely deprotected species (longer migration times). The resolution capacity of CE using a matrix optimized for oligonucleotides can extend to the analysis of oligonucleotides in excess of 100 bases. An example of the separation of a standard oligonucleotide mixture ranging from 40 to 60 bases is presented in Figure 10.9.2. Single-base resolution is readily observed.

An increasing number of oligonucleotide applications require modification of the basic oligonucleotide probe—e.g., biotinylation, fluorescent dye modification, phosphorylation, base modification, addition of phos-

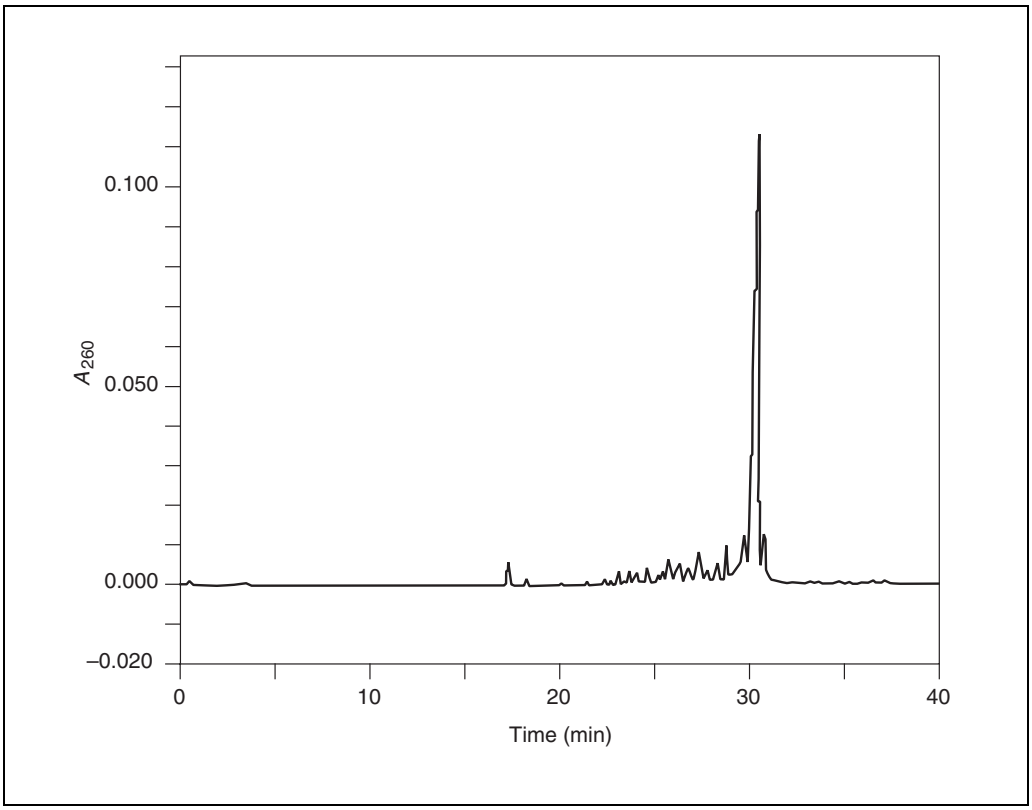

Figure 10.9.4 CE separation of a synthetic 20-mer oligonucleotide. The coupling failure products (shorter migration times) and incomplete deprotection products (longer migration times) are clearly visible.

phorothioate (antisense) backbones, DNA-RNA hybridization, and 5′-amino- or 5′-thiol-modification—prior to conjugation to other molecular species. In all instances, the extent of modification to produce these unique oligonucleotides can be readily assessed by CE due to its high resolution. Recent developments in the area of quantitative gene expression measurements (Freeman et al., 1999) and single nucleotide polymorphism (SNP) detection have utilized the principle of fluorescence energy transfer via oligonucleotide probes. Assays based on quantitative RT-PCR have been developed—e.g., TaqMan (Applied Biosystems), Invader (Third Wave), and Black Hole Quenchers (BioSearch Technologies)—that require either paired single-labeled or dual-labeled oligonucleotides. These reagents are readily analyzed for purity by CE using Basic Protocol 1. CE is particularly useful in this instance since MALDI mass spectrometry (*UNIT 10.1*), a commonly used alternative, fragments the fluorescent label, resulting in multiple molecular species.

Small interfering RNAs (siRNA) can be used for the down-regulation of individual genes (Elbashir et al., 2001). These molecules all share the same features: 2 thymidine deoxynucleotides at the 3′ end followed by 19 ribonucleotides, terminating in a 5′-hydroxyl. These siRNA probes are readily characterized for purity by CE using Basic Protocol 1.

The development of matrices to extend this single-base resolution to ssDNA that ranges in size from 20 to 1000 bases has allowed for development of important applications in the field of automated fluorescence-based DNA sequencing. The most commonly performed sequencing chemistry is the "dye terminator" chemistry, in which the sequence-terminating dideoxy nucleotide also contains the fluorescent reporter group. Consequently, the sequence ladder is labeled in the 3′–hydroxyl position. This means that sequencing reactions can be performed using a primer of any sequence. The alternate "dye primer" chemistry employs a primer that is labeled at the 5′-hydroxyl and is restricted to a small number of commonly used vector sequences—e.g., M13 forward and reverse, T3, T7, and SP6 sequences. An example of this type of application is shown in Figure 10.9.5. Significant effort is being directed towards the development of matrix formulations that will further extend the length of the current sequence reads. Commercial CE sequencing instruments all have shorter run times than slab gel–based automated sequencers and retain comparable sensitivity and accuracy. Each of these applications requires the analysis of ssDNA and is performed under dissociating conditions in the presence of a flowable matrix.

The sizing of larger fragments that have been amplified from genomic DNA has proven to be a very effective method for studying genetic variability in populations. These fragments can be analyzed by capillary gel electrophoresis either under dissociating conditions (short fragments, high size accuracy) or as dsDNA (large fragments, lower resolution). Genomic DNA from eukaryotes contains a large number of tandem-repeating sequences that vary in size from 2 to several dozen bp in size. This polymorphism can be used to advantage when studying human identity or individual heredity. The smaller repeats, commonly referred to as

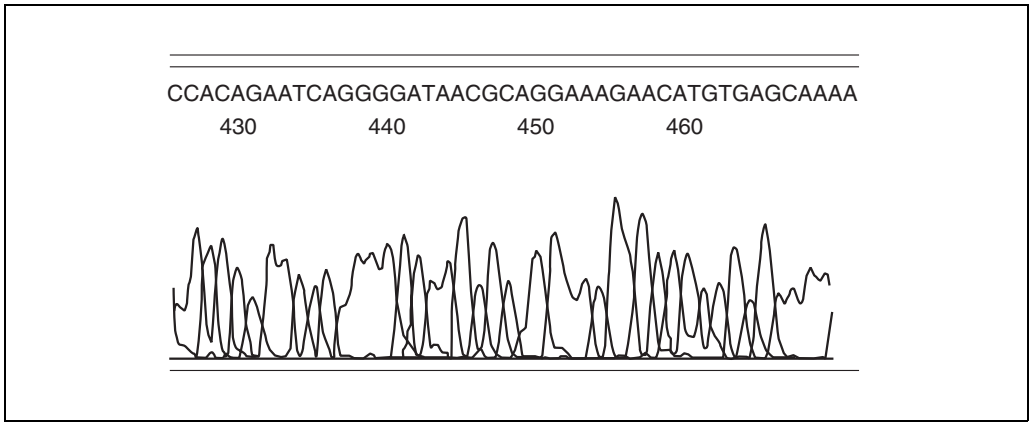

Figure 10.9.5 A portion of the chromatographic output from a PE Biosystems model 373A DNA sequencer using dye terminator sequencing chemistry. Although shown in black and white here, the direct output is color-coded to more clearly illustrate the peaks corresponding to each base in the sequence.

microsatellite or short tandem repeats (STR), are best analyzed as ssDNA (Butler et al., 1994). To determine identity or heredity it is necessary to accurately determine the number of copies of (2- to 5-bp-long) identical sequence repeats in the selected fragment. Fragment lengths are usually <300 bp and require single-nucleotide resolution.

Larger repeats are commonly referred to as variable number of tandem repeats (VNTR) and produce larger-sized fragments that are analyzed as dsDNA. The sizes of the repeats are 10 to 12 nucleotides and up, and fragment sizes can exceed 4000 bp. The required resolution for this type of separation is on the order of 4%.

In contrast to the accurate sizing of DNA, fragment pattern matching can also be a very useful analytical tool. The loss or gain of a restriction site between individuals is sufficiently common that restriction fragment length polymorphism (RFLP) analysis can demonstrate individual identity to a high degree of certainty (Ulfelder et al., 1992). These patterns are readily analyzed by CE. RFLP has found application in the fields of forensic medicine and pedigree testing. Where a polymorphic mutation produces a detectable phenotype, it can also be used as a diagnostic for inherited diseases. It requires the separation of dsDNA in the presence of an intercalating dye for visualization. The matrix is generally a flowable polymer, and fragment lengths are from hundreds to thousands of bp in size.

Mutation detection can also be performed using ssDNA. A denatured DNA fragment can adopt a sequence-specific conformation upon refolding, which will affect its electrophoretic mobility. This property is taken advantage of in single-stranded conformational polymorphism (SSCP) separations (Ren et al., 1997). The DNA region of interest is PCR-amplified from genomic or cDNA to give sufficient copies of a small (<300-bp) fragment. The strands are separated and allowed to refold. The wild-type and mutant fragments adopt different conformations and are resolved with single-base-pair resolution by CE using a nondissociating medium and a flowable polymer matrix.

CE can accurately quantitate and size DNA fragments (Rossomando et al., 1991; Fasco et al., 1995). This can be of considerable value when used to quantitate levels of viruses and pathogenic bacteria. The difficulty arises when quantitation is attempted in the presence of high levels of other DNA, RNA, and proteins. Fluorescence detection coupled with PCR—which can amplify very low levels of DNA in a highly specific manner—can be used to surmount this problem. This amplification can be combined with the inherent sensitivity of CE through the incorporation of fluorescently labeled primers into the amplified DNA. In this fashion samples such as blood can be analyzed at high dilution, thereby reducing the levels of interfering substances to manageable levels. RNA can be amplified by RT-PCR to give the dsDNA fragment that is subsequently quantitated. This is ideal for measuring low levels of viral messenger RNA, i.e., viral load. The fragments are <1000 bp and separations are usually performed under nondenaturing conditions. The use of appropriate quantitative calibration standards is essential. One such standard, a sizing ladder, is shown in Figure 10.9.3, and an example of this technique is presented in Basic Protocol 2.

A majority of CE separations are performed at the standard alkaline pH in the presence of borate, which is an effective buffer in this pH range. The buffer may also contain 6 to 8 M urea, a denaturant that keeps the DNA in its simple single-stranded conformation when required. The urea is omitted from the buffer for analyses where secondary structure plays an important role in the separation, e.g., single-nucleotide polymorphisms or conformational polymorphisms.

The type of matrix that is selected for the actual separation can dramatically influence the quality of the separation that is achieved for a given application. Instrument manufacturers frequently supply kits that have been optimized for a particular application (see Table 10.9.2). These can be very helpful as a first, and maybe the only step required for optimizing individual applications. In general, cross-linked polyacrylamide that is polymerized inside, and covalently attached to, the capillary is best suited for smaller-fragment separations. However, since the column is reused multiple times, many things can reduce resolution—for example, capillary plugging, bubble formation, or drying of the capillary end—and require that the capillary be discarded. In the absence of such external parameters, the lifetime of the capillary is ultimately dependent on the breakdown of the polyacrylamide (or other hydrophilic polymer) matrix.

Flowable polymers have the advantage of wide fragment-separation ranges. These polymers can have a variety of chemical origins (see Table 10.9.2) and have the advantage of being replaced after each separation. This is achieved by applying pressure to the inlet end of the

Table 10.9.2 Supplies and Kits for CE Systems

Manufacturer	Part number	Supplies and kits	Application information
Agilent	192-1311	μPAGE-10 (10% T, 0% C) 100-μm i.d.	
	192-5211	μPAGE-5 (5% T, 5% C) 75-μm i.d.	
	192-3211	μPAGE-3 (3% T, 3% C) 75-μm i.d.	
Amersham	25-6001-0	MegaBACE SNuPe genotyping application kit	Single-base primer extension for SNP analysis
Applied Biosystems	402838	POP-4	Gel for microsatellite, SNP, differential display, AFLP, and other genotyping applications using capillary #402839 and buffer #402824 on the 310 platform
	4316355	POP-4	Gel for microsatellite, SNP, differential display, AFLP, and other genotyping applications using capillary array #4315930 and buffer #402824 on the 3100 platform
	4313087	POP-5	Gel for high-throughput DNA sequencing on the 3730 platform
	402844	POP-6	Sequencing applications on the 310 platform, including template suppression reagent (TSR) for 67-cm capillary #402840 and buffer #402824
	4316357	POP-6	Sequencing applications on the 3100 platform with 50-cm capillary #4315930 and buffer #402824
Beckman Coulter	477480	eCAP ssDNA 100-R kit, including gel, caps, and standard	Oligonucleotides, RNA, and antisense DNA from 10 to 100 bases
	477407	eCAP dsDNA 1000 kit	Analysis of dsDNA fragments from 72 to 1000 bp
	477486	eCAP dsDNA 20,000 kit	Analysis of dsDNA fragments from 1,000 to 20,000 bp
	477409	EnhanCE Dye	Intercalating dye for LIF applications

capillary. However, some of these polymers are quite viscous and require considerable pressure within the instrument (up to 1600 psi) to load the capillary. Linear polyacrylamide, which contains no crosslinker, is a flowable polymer that can produce a very effective sieving matrix. The sieving properties are dependent upon both the polymer concentration and the average chain length (molecular weight). In general, lower polymer concentrations of higher average molecular weight are preferred for the separation of high molecular weight DNA, the reverse being true for lower molecular weight DNA. The selection of an optimal combination of these conditions must be balanced by viscosities that are compatible with the pumping capabilities of the CE instrumentation. It is essential to avoid purchasing incompatible components by matching the instrument capabilities to the matrix that has been selected for the given application. With bare fused silica, either cellulose-based gels (e.g., hydroxyethylcellulose; Aldrich) or acrylamide-based gels (PE Biosystems) can be used. Replacing coated or gel-filled capillaries can be quite expensive; as premature failure of the capillary can normally be attributed to excessive voltage during separation or to inappropriate storage, it is worthwhile to take precautions for avoiding these problems. All manufacturers of CE instrumentation offer UV absorbance detection systems that will work for most general applications. Beckman Coulter and Bio-Rad offer laser-based fluorescence detectors for high-sensitivity CE applications. PE Biosystems offers a single-capillary laser fluorescence instru-

ment for genetic analysis and DNA sequencing, while Beckman Coulter offers similar instrumentation in an eight-capillary format.

Critical Parameters

Oligonucleotide purity

The most common size of synthetic oligonucleotide probes is in the range of 20 to 30 bases. However, some applications require synthetic probes that are 100 bases or more in length. Single-base resolution over this range is essential in order to obtain an accurate assessment of purity. The salt content of the oligonucleotide should be kept to a minimum (<50 mM) in order to obtain optimal resolution. The oligonucleotide should be dissolved in water, or serially diluted in water from the stock TBE solution, prior to electrophoresis. Concentrations should be in the 1 µg/20 ml range for UV absorbance detection. The presence of alkaline-pH buffers and urea in the separation matrix and electrophoresis buffer are essential for single-stranded separations. It is important to run a standard—poly$(A)_{40\text{-}60}$—at the beginning of each set of analyses in order to confirm that the electrophoretic resolution is optimal (see Fig. 10.9.2).

DNA sequencing

In addition to the items above, this application requires single-base resolution over the range of 20 to 1000 bases. Automated, DNA sequencing instruments rigidly control the separation conditions to minimize temperature and power fluctuations and to eliminate variability in the matrix loading protocols. Consequently, most of the critical parameters are associated with template and primer quality and quantity issues. High-quality, salt-free plasmid, cosmid, or PCR-derived DNA is essential. The optimal molar ratio of template to primer is 1:1 and an imbalance of either component outside a ratio of 4:1 will give unusable sequences. Template concentrations should be in the 1 µg/20 µl range. The presence of proteins, salts, detergents, etc. in the template can inhibit the DNA polymerase and kill the reaction. It is essential to remove the excess fluorescent primers or dideoxynucleotides before separating the sequencing ladder on the CE. Further details on the essential parameters of the sequencing reaction itself can be found in Ausubel et al. (2003).

Fragment sizing

Selection of an appropriate separation matrix is probably the most important issue when analyzing microsatellite repeats. Resolution of the smaller fragments at the one to two nucleotide level is required. The DNA should be single-stranded and separated with denaturants. The use of internal size standards is essential since the absolute mobilities can change from run to run and from sample to sample. When analyzing the VNTR fragments, which have larger repeating units, base pair resolution in the 3% to 6% range is required. These fragments are analyzed as double-stranded DNA without denaturants. Since the fragments are longer, lower matrix concentrations are preferred. Again, internal standards are essential.

The fragments are either obtained directly from genomic DNA, or PCR-amplified DNA. Considerable care has to be taken to remove particulate matter and salts from these samples prior to CE separation. The internal diameter of most capillaries is only 50 to 100 µm, and they are sensitive to plugging. Sometimes plugged capillaries can be salvaged by high-pressure back-flushing, but replacement is usually necessary. Salt concentrations should be <50 mM to minimize sample loading problems.

RFLP mutation screening

The same criteria for VNTR analysis are applied to mutation screening—the major exception being that the application requires pattern matching between control and mutant DNA, rather than absolute sizing. The double-stranded fragments must assume their sequence-dependent conformations. The addition of 10% glycerol to the flowable polymer significantly improves the discrimination of the electrophoretic separation.

Quantitation (RT-PCR)

The readout from this application is the absolute level of viral RNA in a biological sample. The PCR amplification is specific, but the target is present in a mixture of other nucleic acids, proteins and salts. The use of a fluorescent label or the addition of an intercalating dye, such as YO-PRO-1 or Sybr Green I, as well as the ability to dilute out the interfering substances, are essential features of this application. The fragments are separated as double-stranded PCR products and are quantitated by peak height on the electropherogram. The presence of salts in the starting material and in the PCR reaction can cause variable sample loading if electrokinetic sample injection is used. Sample loading by pressure injection bypasses these problems.

Absolute quantitation of the PCR product has to take into account both the length of the DNA and the degree of incorporation of the intercalating dye. This is achieved by coinjecting a standard of known concentration with the sample. If this standard contains a ladder of fragments of known length then the quantity of the amplicon can be accurately determined. This procedure carries the added advantage of confirming the correct fragment size.

The difficulties encountered with this type of quantitation are not associated with the CE quantitation but with the PCR amplification step. It is essential that a dilution series is performed on the PCR mix to ensure that the reaction is linear with respect to cycle number. In the case of competitive PCR, it is essential that the competing template concentration approximates that of the target template. Alternatively, expression levels can be quantitated relative to a "housekeeping" gene. This is a gene, such as β-actin, that is constitutively expressed in the cells of interest. In this procedure the β-actin gene is coamplified with the gene of interest prior to quantitation by CE. As indicated previously, this field has recently undergone major expansion. The introduction of automated PCR-based instrumentation and the availability of high-quality fluorescently labeled oligonucleotides has produced a number of robust RT-PCR-based kits. They have superseded the intercalating dye approach for some applications.

Troubleshooting

Mechanical and electrical problems that might be encountered during instrument use are addressed in the troubleshooting sections of the manufacturers' manuals. One difficulty is the loss of electrical continuity during a run. This loss of current can be caused if a small bubble forms in the column during injection or if the power generated by the run is so great that the solution boils or outgasses. Purging the column after a failed run removes any bubbles in the column. The vial containing the sample must have sufficient liquid to cover the end of the column during injection to prevent introduction of air into the column. On the autosampler, the vials can dry out during a long run, so sample temperature control or the use of the correct cap on the vial is required to slow evaporation. Reducing the buffer concentration or the run voltage can eliminate bubble formation during a run. Degassing the buffer is also useful if the outgassing problems continue.

On occasion capillaries can become plugged. This is normally due to the capillary matrix being allowed to dry out. When not in use, the capillary can remain installed on the CE instrument, but both ends must remain submerged in buffer. If it is removed from the instrument the capillary should be stored refrigerated, with both ends capped to prevent evaporation. The capillary can also become plugged from insoluble material that is present in the sample. Sometimes it is possible to apply pressure to the anode outlet and blow out the plug. More likely, however, the capillary will need to be replaced.

Loss of resolution on a separation of standards can have several causes: e.g., the buffer, the separation matrix, or the capillary. A process of elimination should follow the course of first trying fresh buffer, then new matrix, and finally changing out the capillary. Where separation of standards is normal, but resolution or signal strength is poor, the cause is likely excess salts and/or buffers in the samples. Examining the behavior of the electrical current during the run will probably assist in troubleshooting. Most CE instruments simultaneously monitor both detector output and current flow throughout the electrophoresis run.

A drop in sensitivity or signal strength can be observed when multiple injections are made from a single standard vial (especially if the standard is dissolved in deionized water). This phenomena can be due to salt contamination of the sample by residual matrix buffer on the outside of the capillary (Guttman and Schwartz, 1995). Keep in mind that any source of additional ions to the sample (e.g., salt, buffer, or other ion species) will affect an electrokinetic injection. The ions of the sample will be effectively diluted by the ions from the contaminant. Sample preparation to remove variable salt contamination will improve the consistency of injections (Ruiz-Martinez et al., 1998).

Anticipated Results

The separation of oligonucleotides should show single-base resolution from 5 bases to >100 bases. The synthetic efficiency of the assembly process is in excess of 99% (Pon et al., 1996), such that the yield of full-length product for a 100-mer should still exceed 30% of the total DNA present. For a 25-mer the yield of full-length product should exceed 80%. The addition of various functional moieties to the oligonucleotide will normally be seen as an increase in size (or decrease in mobility). The

addition of a group as small as a phosphate group at the 5′-hydroxyl will make the oligonucleotide appear longer by a single base. The size increase, however, is insufficient proof of successful modification. This must be verified by obtaining a molecular weight by mass spectrometry (e.g., MALDI-TOF; *UNIT 10.1*).

The DNA-sequencing application (as stated previously) can have single-base resolution up to 1000 bases or more, though performance today on commercial multicapillary instruments is ~600 bases or more in 2 hr. The smallest sequencing fragments will be detected as sharp, tight bands usually ~1 to 3 sec in width. The larger (slower-moving) fragments will show considerable band broadening due to diffusion as well as reduced separation efficiency of the matrix.

The microsatellite and VNTR analyses will show well-resolved peaks for the various multimers that are reflective of the number of repeats in the sample. These should be well resolved from the sizing control that has been added to, or coinjected with, the sample of interest.

The variability of these repeats at a given locus should give sufficient information to clearly identify relatedness between individuals. The expression levels of various isotypes should be sufficiently well discriminated to use this technique as a potential diagnostic test for disease states.

Quantitative PCR data should be sufficiently accurate to detect a 3- to 5-fold change in viral load level. The linearity of response of the standard and target DNAs should extend to the low to sub-nanogram level.

Time Considerations

These will vary dependent upon the type of separations that are required. Preparation of the stock solutions can take up to 1 hr. Filling the capillaries with fresh gel can take 1 hr. The analysis of synthetic oligonucleotides is normally completed within 30 min and sample preparation time is minimal. The DNA sequencing application probably requires the longest separation times. A 2-hr cycle time from injection to injection is normally sufficient to obtain 500 to 600 bases of high-quality sequence. Regeneration and loading times are <15 min. Preparing the stock solutions from the kit takes 24 hr. Filling the capillary with gel takes 30 min. Analysis of microsatellite fragments can take as little as 15 min, whereas the analysis of VNTR fragments takes up to 30 min. Regeneration times are <10 min. The analysis component of quantitative PCR is similar. However, this application requires multiple runs in order to obtain linearity curves for the PCR products and the calibration standards.

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Key Reference

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An excellent reference on all aspects of capillary electrophoresis separations. Chapter 11, on DNA, goes into much greater depth than is possible here on the theory of separation, selection of buffers, and selection of gel matrices.

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