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Review

# Analysis of clinically relevant, diagnostic DNA by capillary zone and double-gradient gel slab electrophoresis

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**Abstract**

A number of applications of capillary zone electrophoresis (CZE) in sieving liquid polymers (notably linear polyacrylamides and cellulose) for the analysis of polymerase chain reaction products of clinically relevant, diagnostic DNA, are reviewed here. The fields covered are human genetics, quantitative gene dosage, microbiology and virology, forensic medicine and therapeutic DNA (notably antisense nucleotides). Some unique, novel developments are highlighted, such as (a) non-isocratic CZE, i.e., temperature-programmed CZE for detection of DNA point mutations and (b) the synthesis of novel N-substituted acrylamides, offering extreme resistance to alkaline hydrolysis, coupled with high hydrophilicity. In the field of denaturing gradient gel electrophoresis (DGGE), as routinely performed in gel slabs, a novel methodology is described, i.e., double-gradient DGGE. In this technique, two gradients are simultaneously applied along the migration direction; a chemical denaturing gradient, for partially unwinding homo- and hetero-duplexes of DNA and a porosity gradient, for re-compacting diffuse bands melting over a broader range of denaturing conditions. Both the CZE and the slab gel methodologies, with the latest developments described in this review, appear to be promising tools for screening diagnostic DNA. 

*Keywords:* Genetic disorders; Reviews; DNA

## Contents



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## 1. Introduction

Small alterations in the DNA sequence of genomic material lead to many human diseases, such a cancer, diabetes, heart disease, myocardial infarction, atopy, atherosclerosis, cystic fibrosis, Alzheimer's disease, Duchenne's muscular dystrophy and thalassemias. These alterations in DNA sequence include many types of mutation and polymorphism in genomic material, such as one or several nucleotides substitutions, deletions or insertion of some sequence, differences in the variable number of tandem repeats (VNTR), and the genomic instability of microsatellite repeats [1]. DNA diagnosis for human diseases thus has very important applications in the fields of genetic and medical research, clinical chemistry and forensic science. Since a large proportion of sequence variations in the human genome are caused by single base changes, any method used to detect mutations or polymorphism must be capable of detecting single-base substitutions.

The currently available methods for mutation and polymorphism analysis include some modified polymerase chain reaction (PCR) techniques [2], restriction fragment length polymorphism (RFLP) [3,4], single-strand conformation polymorphism (SSCP) [3], VNTR [5], microsatellite analysis [6], hybridization techniques [7], denaturing gradient gel electrophoresis (DGGE) [8], temperature gradient gel electrophoresis (TGGE) [9], heteroduplex analysis (HA) [10] and chemical mismatch cleavage (CMC) [11]. Some recent reviews rate the merits and limits of the various techniques [12–16].

Although most of the above techniques have been developed and are used primarily in the slab gel format, capillary zone electrophoresis (CZE) is now emerging as a competitive analytical tool for separating a variety of charged and uncharged molecules, including proteins and nucleic acids [17]. Its advantages comprise minute sample requirements (sample zones of just a few nl), extremely high sensitivity (of the order of yocto-moles with laser-induced fluorescent detection) and on-line peak detection and integration. The applications in the field of nucleic acid analysis range from DNA sequencing [18,19] to pulse fields for large DNAs [20]. Some reviews have already appeared describing the use of CZE for the analysis of PCR-amplified fragments and for the detection of genetic defects [21–23].

In this review, we will give an up-to-date view of the progress made to date in the analysis of genetic diseases, including detection of microbiological and viral DNAs related to human health, quantification of therapeutic DNAs, such as antisense oligonucleotides, and analysis of genetic polymorphisms that are involved in forensic medicine. In addition, we will

highlight some important developments that have improved the performance of both CZE and slab gel operations. One is the possibility of performing CZE in a non-isocratic mode, i.e. under temperature-programmed conditions (called TGCE, thermal gradient capillary electrophoresis) [24–26]. As a fundamental distinction, unlike TGGE, where the temperature gradient exists along the separation space and is controlled externally via circulating liquid and thermostats, the denaturing temperature gradient in the fused-silica capillaries is generated internally, via ohmic heat produced by voltage ramps and exists only in time, not in the separation space. The other, related to slab gel operations for spotting point mutants, is the possibility of running PCR-amplified fragments, in the classical DGGE mode, not against a single, but against a double gradient [91]: The first gradient (of denaturant, typically urea and formamide, but also temperature) is needed to partially unwind the homo- and hetero-duplexes and thus form the characteristic spectrum of four bands, indicative of the presence of a single base substitution in the DNA filament. The second gradient (colinear with the first) is a porosity gradient and is needed to re-compact the zone of homo- and (especially) hetero-duplexes, which often produce smeared and diffuse bands, due to melting over a not-so-narrow range of denaturant milieu. With these novel methodologies, we feel that both CZE and slab gel electrophoresis now offer substantially improved performance, which will surely be felt in clinical chemistry laboratories, in forensic medicine and in the field of genetic counselling.

### *1.1. CZE for the analysis of clinically relevant, diagnostic DNA*

Table 1 summarizes what we have been able to find in the literature so far. We will briefly highlight some aspects of general interest in the various cases.

### *1.2. Cystic fibrosis (CF)*

CF is the most common autosomal recessive disease among Caucasians, with an incidence of 1/2500 of live-born individuals and a carrier frequency of 1/25 individuals. Since the CF conductance transmembrane regulator (CFTR) gene, responsible for the disease, was identified, more than 600 mutations have been discovered, together with a number of sequence variations and polymorphisms. The most common mutation, ΔF508, a three-base-pair deletion that abolishes the Phe codon in position 508 of the expressed protein, is detected quite easily by amplifying a 98-bp fragment in exon 10 in normal individuals vs. a 95-bp fragment in CF carriers. This deletion is dealt with in refs. [28–30]. In ref. [27], three additional mutations are simultaneously separated: G542X, 1717-1G→A and N1303K. This analysis of four different pathological conditions in a single test allows 65% of the molecular defects in CF patients to be dealt with. In ref. [29], an additional (besides the ΔF508 and G542X) three mutations are screened: W1282X, G551D and 621+1G>T, produced by a PCR–ARMS (amplification refractory mutation system) technique, producing fragments ranging in size from 160 to 367 bp. Interestingly, in ref. [30], an additional CF mutation (3905 insT in exon 20 of the CFTR gene), producing two fragments of 105 bp (normal) and 106 bp (mutant), can be efficiently screened with almost baseline resolution for the 105/106 bp pair. In ref. [31], screening for CF conditions is proposed via linkage analysis that exploits GATT microsatellites located at the junction of intron IVS6a and exon 6b. It consists of two main allelic forms, one hexameric and the other heptameric, with a 41% frequency of observed heterozygosity. In this VNTR analysis, it is found that most patients are heterozygous, carrying both the exameric (111 bp) and the heptameric (115 bp) repeat alleles [31]. In refs. [24–26], a variety of point mutants in CF are detected by the TGCE technique, which was mentioned briefly in Section 1. In a way, this technique is analogous to temperature sweep gel electrophoresis, first reported by Yoshino et al. [32] and subsequently modified by Penner and Bezte [33], but with substantial innovations. Not only the temperature gradient is generated from within the capillary, via ohmic heat generated by voltage ramps, but, additionally, our overall $\Delta t$ is rather minute (1 to 1.5°C) and the sweep rate is very gentle (e.g., 0.05°C/min). On the contrary, in refs. [32,33], huge temperature intervals are applied (e.g., from 45 to 63°C or from 25 to 55°C) and, often, stepwise gradients are adopted (e.g., sudden increments of 10°C during each hour of electrophoresis).

Table 1
Survey of selected capillary electrophoretic separations of clinically relevant diagnostic DNA

| Disease | DNA amplification | Sieves | Detection | References |
|---|---|---|---|---|
| *Human genetics* | | | | |
| Cystic fibrosis | Allele-specific PCR and restriction digest of PCR products (deletion) | 6% linear PAA | UV 254 nm | [27] |
| Cystic fibrosis | PCR, ΔF508 | 6% linear PAA | UV 254 nm | [28–30] |
| Cystic fibrosis | PCR/GATT microsatellites | 6% linear PAA | UV 254 nm | [31] |
| Cystic fibrosis | Point mutants, TGCE | 8% linear poly(AAEE) | UV 254 nm | [25,26] |
| Duchenne/Becker muscular dystrophy | PCR multiplex reaction | 6–10% linear PAA | UV 260 nm | [35] |
| Dystrophin gene | RFLP | 0.5% HPMC | UV 254 nm | [3] |
| Thalassemia | Point mutants, TGCE | 4% poly(AAP), 1.5% HEC | UV 254 nm | [42] |
| Congenital adrenal hyperplasia | PCR deletion | 6% linear PAA | UV 254 nm | [45] |
| Androgen insensitivity syndrome | CAG triplet analysis | 6% linear PAA | UV 254 nm | [47] |
| Kennedy's disease | CAG triplet expansion | 8% poly(AAEE) | UV 254 nm | [48] |
| N-ras gene (human cancer) | Point mutants SSCP | 8% PAA | UV 260 nm | [49] |
| ERBB2 oncogene | RFLP | 0.5% HPMC | UV 260 nm | [50] |
| TX gene | PCR | 3% PAA | LIF | [51] |
| p-53 | SSCP | 4% PAA | UV 260 nm | [52] |
| p-53 | SSCP | 2% PAA | LIF | [53] |
| Cancer (microsatellites instability) | PCR | Bio-Rad sieving polymer | UV 260 nm | [54] |
| Apolipoprotein B gene | PCR/VNTR | 0.7% MC | UV 260 nm | [55] |
| Apolipoprotein E gene | PCR/RFLP | 3%T PAA | UV 260 nm | [56] |
| Apolipoprotein E gene | PCR/RFLP | Beckman e/CAP | LIF | [57] |
| Medium chain acyl CoA dehydrogenase deficiency | PCR/allele specific | Polyacrylamide gel | LIF | [58] |
| von Willebrand Factor gene | PCR/VNTR | 1% HEC | LIF | [59] |

Table 1. Continued.
Survey of selected capillary electrophoretic separations of clinically relevant diagnostic DNA

| Disease | DNA amplification | Sieves | Detection | References |
|---|---|---|---|---|
| Fetal DNA (Y-chromosome) | PCR | Beckman ds DNA 1000 gel buffer | LIF | [60] |
| *Quantitative gene dosage* | | | | |
| Down's syndrome | Quantitative PCR | 8% PAA | UV 254 nm | [61] |
| Rh D/d genotyping | Quantitative PCR | 8% PAA | UV 254 nm | [62] |
| Follicular lymphomas | Competitive PCR | 4% PAA | UV 260 nm | [63] |
| Basic fibroblast growth factor | Competitive RT-PCR | 6% PAA | UV 254 nm | [64] |
| *Microbiology/virology* | | | | |
| *Mycobacterium tubercolosis* | SSCP and dideoxy fingerprinting (ddF) | 1% HEC or 3%T, 0.5%C PAA gel | LIF | [66] |
| Hepatitis C virus | RT-PCR | 1% HEC | LIF | [67] |
| Polio virus | RT-PCR | 3%T linear PAA | UV 254 nm | [67] |
| HIV-1 | RT-PCR | 3% linear PAA | LIF | [68–70] |
| *Forensic medicine* | | | | |
| Mitochondrial DNA | PCR | 1% HEC | LIF | [71] |
| Mitochondrial DNA | PCR | 0.5% MC | LIF | [72] |
| VNTRs at locus D1S80 | PCR/VNTR | 0.5% HEC | LIF | [73,74] |
| VNTRs at locus D1S80 | PCR/VNTR | 0.5% MC | LIF | [75] |
| VNTRs at locus HUMTH01 | PCR/VNTR | 1% HEC | LIF | [76,77] |
| VNTRs at locus HUMTH01 | PCR/VNTR | 3%T, 3%C gel | UV 260 nm | [78] |
| *Therapeutic DNA* | | | | |
| Antisense oligonucleotides | | 18% PAA | MALDI–MS LIF | [83–85] |
| Antisense oligonucleotides | | 10% PAA, isoelectric His | UV 254 nm | [88] |
| Antisense oligonucleotides | | 10%T PAA, pH gradient | UV 254 nm | [89] |

We have, moreover, developed a dedicated software package that allows us to precisely link the inner capillary temperature to the experimental parameters [such as capillary diameter, its total length, the electric current values (μA) linked to a given applied voltage, the buffer's electric conductivity and its thermal coefficient of conductivity] [34]. In [24], our TGCE technique was proven to be suitable for low CF melters (in the 45–49°C interval); in [25], the TGCE method was extended to intermediate- and high-melters (up to 65°C), thus confirming the wide applicability of our approach. It should be noted,

additionally, that since we work with a background electrolyte containing 6 *M* urea, these $T_m$ values should be increased by 12°C, since it has been estimated that the apparent $T_m$ is lowered by 2°C/mole (of added urea).

### *1.3. Duchenne's muscular dystrophy (DMD)*

DMD is an X-linked, recessive disorder, resulting from mutations in the dystrophin gene. It affects about 1 in 3500 newborn males and it is characterized by progressive muscle degeneration, leading to death in the second or third decade of life. Becker's muscular dystrophy (BMD) is a clinically less severe form of myopathy, allelic to DMD. In the late 1980s, two PCR assays were described by Chamberlain et al. [93] and by Beggs et al. [94], which allow one to detect over 98% of DMD/BMD deletions. Each of these are based on specific co-amplification of nine dystrophin gene exons, leading to direct identification of deletions. Due to their efficiency, reliability and ease of use, they represent a very useful method for the DNA-based diagnosis of DMD/BMD. Since each of them is based on specific co-amplification of nine dystrophin gene exons, a method attempting to simultaneously analyze DMD/BMD should offer unambiguous resolution and identification of eighteen DNA fragments ranging in size from ca. 100 to 500 bp. Gelfi et al. [35] have developed a novel capillary electrophoresis method that allows the simultaneous analysis of the two PCR sets with full diagnostic values. It consists of (a) an ultra-stable inner capillary coating that is based on a novel acrylamido monomer (N-acryloyl amino ethoxy ethanol) [36] (AAEE; and more recently, on acryloyl amino propanol, AAP) [37]; (b) a very low viscosity (barely 70 mPa) sieving polymer solution, formed by short-chain (average $M_w$ of 230 000, 55 000 $M_n$) polyacrylamides [38]. The set of eighteen fragments comprises the following: 88, 113, 139, 154, 170, 196, 202, 238, 268, 271, 313, 331, 357, 360, 388, 410, 506 and 547 bp.

In another approach, Del Principe et al. [39] have used RFLP and CZE to characterize a PCR-amplified product of a 740-bp DNA fragment from the DXS locus of the dystrophin gene. The fragment is then digested with XmnI, yielding polymorphic fragments of 520 and 220 bp, corresponding to alleles A1 and A2, respectively. Individuals that are homozygous for A1 reveal only the 740 bp fragment. Those homozygous for A2 display both the 520 and 220 fragments, whereas heterozygous individuals yield all three fragments.

### *1.4. β-Thalassemias*

The term β-thalassemia refers to a heterogeneous group of disorders, characterized by deficient production of β-globin chains relative to α-globin chains in erythroid cells [12]. The majority of β-thalassemia alleles are caused by single nucleotide substitutions or small deletions or insertions that affect RNA transcription, processing or translation. Over the past few years, over a hundred different β-thalassemia mutations have been described [40]. A large number of these mutations have been shown to be population-specific and the spectrum of these defects has been established in several countries [41]. Gelfi et al. [42] have applied CZE in the TGCE mode to the analysis of a number of point mutations in thalassemias. As shown in Fig. 1, excellent results

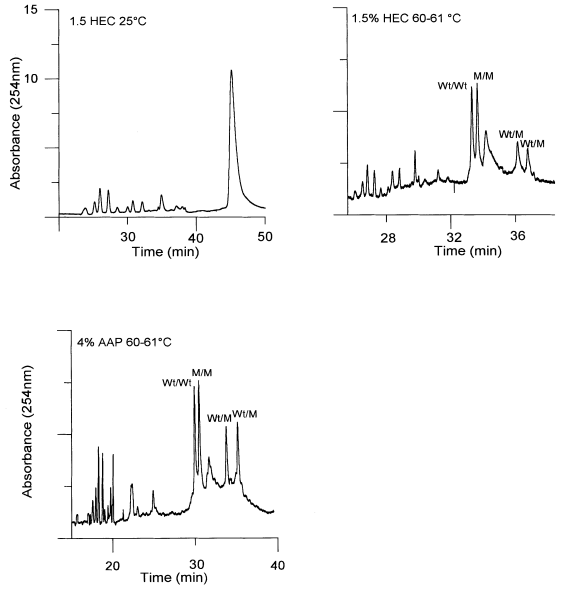


Fig. 1. CZE of fragment IIIC (319 bp), IVS-IInt.1 of the β-globin gene, carrying a G→A transition. (A) control run, at 25°C, in 1.5% HEC; (B) same as (A), but in a temperature interval of 60–61°C; (C) same as B, but in 4% poly(AAP) (from Gelfi et al., [42], reprinted with permission).

(with ample resolution of the spectrum of four bands (the two homo- and two hetero-duplexes) are obtained in both 1.5% hydroxyethylcellulose (HEC) and in poly(AAP), although the latter matrix clearly gives sharper bands, especially in the case of the two hetero-duplexes. The TGCE methodology has been described above, in the case of point mutants in the CFTR gene, and it should be regarded as a unique variant of temperature-programmed gas chromatography [43].

### *1.5. Congenital adrenal hyperplasia*

Congenital adrenal hyperplasia [also called 21-hydroxylase (21-OH) deficiency] is recessively inherited and accounts for over 90% of the genetic disorders of steroidogenesis [44]. The severe form occurs in about 1 in 10 000–14 000 individuals; approximately 70% of classical 21-OH deficiency patients show an inability to conserve dietary sodium (salt wasting), while the remainder have been classified as ''simple virilizers''. The mild, non-classical form (late onset) is more common, affecting 1 in 1000–2000 individuals. The diagnosis of 21-OH deficiency is suggested by genital ambiguity in females, a salt-losing episode in either sex, or by rapid growth and virilization in males. Plasma 17OHP (17-hydroxyprogesterone) is markedly elevated ($>$2000 ng/dl after 24 h of age) and is hyperresponsive to stimulation with adrenocorticotropic hormone (ACTH). Gelfi et al. [45] have proposed a CZE analysis method, consisting of identifying an 8-bp deletion in affected individuals at the P450c21B locus. The PCR protocol allows the amplification of a 135-bp fragment in normal individuals and of the disease-linked, 127-bp fragment. The CZE run is typically performed in 6% liquid linear polyacrylamide in coated capillaries, with detection at 254 nm.

### *1.6. Androgen insensitivity syndrome*

The androgen receptor (AR) is a DNA-binding, transcription-regulating protein. Mutations in the AR block the normal pathway of androgen action (testosterone and 5$\alpha$-dihydrotestosterone) and result in a number of phenotypic abnormalities in male sexual development. A spectrum of different phenotypes is known: The complete androgen insensitivity syndrome (CAIS, also called Morris' disease), the partial androgen insensitivity syndrome (PAIS), the infertile male syndrome and the undervirilized fertile male, each of which is transmitted as an X-linked trait [46]. AR abnormalities have been characterized in cultured genital skin fibroblasts from patients with androgen resistance. These defects span from normal binding, qualitative abnormalities to the complete absence of androgen binding. Cloning of cDNA encoding the androgen receptor has made it possible to elucidate the molecular defects that cause androgen resistance. In most patients, mutations are single nucleotide substitutions within the coding region of the AR, indicating that a heterogeneous mechanism is the cause of androgen resistance. The CAG trinucleotide repeat in exon A was found to be polymorphic and ranged from 17 to 28 Glu residues. This highly polymorphic CAG repeat in exon A represents a useful marker for the determination of affected, unaffected and carrier members within pedigrees showing a recessive X-linked transmission of AIS. Inheritance of polymorphic fragments represents a marker for following transmission of the disease within a family, allowing us to distinguish between the two maternal X chromosomes. Gelfi et al. [47] have proposed a CZE method based on the analysis of CAG triplet polymorphism in families carrying the AIS syndrome. Easy separation was obtained for 139- and 160-bp fragments in families carrying a difference of seven CAG repeats, but even more difficult cases (such as those of families exhibiting fragments of 136- and 139-bp, thus differing by only 1 CAG repeat) were resolved with precision and diagnostic value.

### *1.7. Kennedy's disease*

Nesi et al. [48] have applied CZE to the screening of Kennedy's bulbospinal amyotrophy, an X-linked moto-neurological disorder that is characterized by an increase in the number of triplet repeats (CAG) in the gene-coding region. In this disease, in the 5′ region of the first exon of the AR gene, a range of 40–52 CAG triplet repeats is found, with repeat lengths that are almost double that found in normal individuals. Nesi et al. [48] have amplified two fragments: Normal individuals were found to express

only the 480-bp DNA size; homozygous, affected individuals expressed a 540-bp fragment and heterozygous carriers contained both fragments.

### *1.8. N-ras gene and other oncogenes*

The *N-ras* gene is an oncogene which, when mutated, may induce de-differentiation, resulting in neoplasia. Thus, screening for point mutations in this gene is important for the diagnosis of human cancer. Arakawa et al. [49], in a first round of PCR, have prepared four kinds of *N-ras* gene (149 bp fragments) carrying different mutations at site 181 (C→A, T and G). In a second round of PCR, the same mutations at site 181 were expressed in a 60-bp fragment (C181 being the normal, and A181, G181 and T181 being the various mutations). These 60 bp fragments could be separated (but not to baseline) by SSCP in a capillary filled with 8%T poly(acrylamide) and detected at 260 nm. By the same token, Ulfelder et al. [50] have analyzed the ERBB2 oncogene by the technique of RFLP by CZE in 0.5% hydroxypropylmethyl cellulose (HPMC) with detection at 260 nm. Ren et al. [51] have analyzed PCR products from the Tx gene (also connected with human cancer) by CZE in 3% linear polyacrylamide (PAA) with laser-induced fluorescence (LIF) detection (excitation at 488 nm, detection at 520 nm). Kuypers et al. [52] have screened point mutants in p-53 (in fact, a tumour suppressor gene) by SSCP in a CZE column filled with 4% liquid PAA; two 372 bp filaments, differing in a single nucleotide mutation, could be separated. Katsuragi et al. [53] have investigated additional alterations in exon 7 (both point mutations and small deletions) of the p-53 gene in human lung tumours by SSCP–CZE with LIF detection. Microsatellite instability, associated with carcinogenesis, was studied by Oto et al. [54] in a Bio-Rad proprietary sieving liquid polymer buffer.

### *1.9. Apolipoprotein B and E genes*

The apolipoprotein B (apoB) variable number of tandem repeat (VNTR) alleles containing larger repeat units are a risk factor for coronary heart disease. Baba et al. [55] have resolved, by CZE, some alleles differing by one or two 16 bp repeats in the DNA size range up to 600 bp. ApoB alleles differing in length by two-to-four repeat units were also readily distinguishable in the 600 to 1000 bp range. The same authors [56] have also performed apolipoprotein E (ApoE) genotyping for screening for heart disease by CZE–RFLP analysis. Two DNA fragments (72 and 91 bp) were considered by these authors and used to define three genotypes (E3/3, E3/4 and E4/4) that are potentially connected with the risk of atherosclerosis. However, their data have been challenged as being incomplete by Schlenck et al. [57], who argued that six common genotypes should be detected for the technique to be fully applicable to ApoE genotyping. Schlenck et al. [57] have, in fact, amplified and resolved a whole series of fragments for proper ApoE analysis: 16, 18/19 (unresolved), 33, 38, 48, 72, 81 and 91 bp.

### *1.10. Other genetic defects*

Medium-chain acyl coenzyme-A dehydrogenase deficiency was screened by Arakawa et al. [58] using CZE in a capillary containing a cross-linked polyacrylamide gel and by exploiting LIF detection. The most prevalent mutation (a $Lys^{329}$ to Glu substitution, i.e. an A to G mutation) was detected by amplifying allele-specific DNA fragments of 202 bp (normal) and 175 bp (mutant) in length. This disorder, which shows an autosomal recessive inheritance, is known to be highly prevalent among Caucasians and often mimics a Reye-like syndrome or sudden infant death.

Defects in the von Willebrand Factor gene have been analyzed by McCord et al. [59] via amplification of VNTR alleles by CZE in presence of a cross-linked polyacrylamide gel and LIF detection. Fetal DNA detection has been accomplished by Liu et al. [60] via amplification of Y-chromosome-specific DNA sequences in a CZE system exploiting a Beckman gel proprietary buffer.

## 2. Quantitative gene dosage by CZE

Quantitative PCR, followed by precise peak profiling and integration in a CZE run, can be a formidable tool for gene dosage in the diagnosis of quite a few pathological conditions. Some interesting cases will be discussed below.

### 2.1. Pre-natal diagnosis of Down's syndrome

Free trisomy 21 accounts for about 95% of all cases of Down's syndrome, the leading cause of genetically inherited mental retardation. This birth defect is among the most common genetic diseases, with an incidence of about 1 in 700 births, with an exponential increase in pregnancies in women over 35 years of age. Modern proposals for pre-natal genetic analysis of Down's syndrome consist of isolating DNA from amniotic cells and amplifying a highly polymorphic small tandem repeat region of the chromosome-21-specific D21S11 marker. The PCR-amplified fragments are typically 5′-end-labelled with a green or blue fluorescent reporter and data acquisition occurs on-lane in DNA sequencing slab gels. The following patterns are expected: For normal individuals, one or two peaks in a 1:1 ratio. In the case of trisomy 21, the following patterns are found: Either three peaks in a 1:1:1 ratio or a two-peak profile with a 2:1 gene ratio. Gelfi et al. [61] have developed a CZE system that offers precise diagnostic value by exploiting the intrinsic DNA absorbance at 254 nm. The separation occurs in capillaries that are coated with poly(N-acryloylaminoethoxyethanol, AAEE) and filled with a background electrolyte consisting of 89 m*M* Tris–borate, 2 μ*M* EDTA, 2.5 m*M* ethidium bromide and 8% short-chain, low-viscosity, replaceable, liquid, linear, sieving polyacrylamide.

### 2.2. RhD/d genotyping

A safe and reliable method for determining RhD type (positive or negative) and zygosity (D/D or D/d) could have applications, for instance, in the prediction of the D genotype of a father in couples where there is an RhD-negative woman at risk of fetal alloimmunization. CZE has been proposed by Cossu et al. [62] as a novel, reliable and powerful method for the quantitative evaluation of PCR products. RhD is determined by amplifying a 136-bp region that is common to the RhCcEe and RhD genes and a 186-bp region that is specific to the RhD gene. RhD-positive and -negative samples are identified by CZE in sieving liquid polymers, with direct on-line peak densitometric evaluation by exploiting the intrinsic UV absorbance of the DNA fragments at 254 nm. The CZE method not only allows a rapid and reliable assessment of the RhD type (the presence of both 136-bp and 186-bp fragments indicating an RhD-positive type, the presence of only the 136-bp fragment indicating an RhD-negative type) but also a rapid determination of the zygosity, based on the quantitative expression ratio of the 136-bp/186-bp pair. Thus, a 2:1 peak ratio clearly indicates a D/D homozygous individual, whereas a 3:1 peak ratio provides evidence of a D/d heterozygous individual. A nice example of this application is shown in Fig. 2.

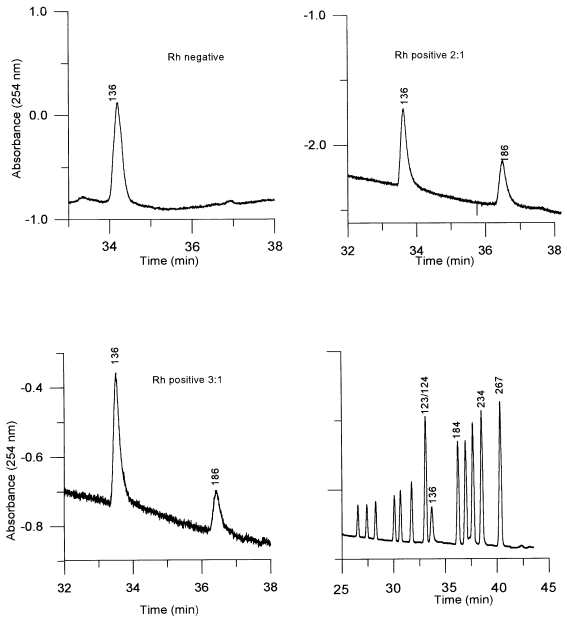


Fig. 2. Rh D/d genotyping and zygosity determination by CZE. Capillaries: 75 μm I.D., 37 cm length (31 cm to the detector), coated with poly(N-acryolyl amino propanol). Background electrolyte: 89 m*M* Tris–borate, 2 m*M* EDTA, pH 8.3, containing 8% short-chain, low-viscosity, liquid sieving polyacrylamide. Sample injection: 15–20 s, at 100 V/cm. Run: 100 V/cm, room temperature. Panel A: RhD-negative individual, exhibiting only the 136-bp peak; panel B: RhD-positive sample; due to the 2:1 peak ratio (136-bp/186-bp) it has been classified as a D/D homozygous individual; panel C: RhD-positive sample; due to the 3:1 peak ratio (136-bp/186-bp) it has been classified as a D/d heterozygous individual; panel D: sizing of the PCR-amplified markers for RhD determination. RhD-negative sample admixed with marker V standards (the 136-bp fragment is clearly visible) (from Cossu et al., [62], reprinted with permission).

### 2.3. Follicular lymphomas

Tumour-specific markers can be used to monitor residual disease in patients during treatment. One such marker is the reciprocal chromosomal translocation t(14;18), which is frequently found in follicular lymphomas (non-Hodgkin's lymphoma). Due to this translocation, the bcl-2 gene on chromosome 18 is coupled to the immunoglobulin heavy-chain locus in chromosome 14. Random nucleotides are inserted between the two genes, providing a patient-specific DNA pattern. By amplification of the translocation breakpoint, in a competitive PCR method, Kuypers et al. [63] could quantify the residual presence of such lymphoma cells during treatment in different patients.

### 2.4. Basic fibroblast growth factor

The local synthesis of polypeptide growth factors (such as the basic fibroblast growth factor, bFGF) might be an important autocrine mechanism to stimulate cell proliferation both in normal and in neoplastic cells. In particular, bFGF is a potent mitogen for a variety of mesoderm- and neuroectoderm-derived cells, including endothelial cells. It was originally isolated from the bovine pituitary, but it is now clear that it can be synthesized by many normal and malignant tissues. Human ovarian granulosa cells and human ovarian tumour epithelial cells are sites of bFGF synthesis. In these tumours, the presence of mRNA coding for the bFGF was demonstrated by reverse transcription (RT) PCR. For quantitative purposes, competitive PCR is adopted, using a sequence of a highly homologous bovine bFGF as the competing fragment. By using RT-PCR, Gelfi et al. [64] reported a CZE protocol, in 6% linear, liquid polyacrylamide, that was able to fully resolve and quantify the undigested (354 bp) bovine and the digested (295 and 59 bp) human fragments, with peak ratios that were in good agreement with autoradiographic data obtained by polyacrylamide gel electrophoresis (PAGE) in slab gels.

## 3. Microbiology/virology

A number of interesting applications of CZE have been proposed for the detection of bacterial and viral infections, of potential use in clinical diagnosis.

### 3.1. Mycobacterium tubercolosis

Felmlee et al. [65] applied CZE to the detection of point mutations in a strain of *Mycobacterium tubercolosis*, with the aim of rapidly identifying those organisms harbouring a mutation that could be associated with resistance to a specific drug, rifampin. Such mutants could indeed be detected by CZE techniques exploiting both SSCP and dideoxy fingerprinting.

### 3.2. Hepatitis C virus

An ideal example of DNA-based assays for use in clinical diagnosis is the screening of sera of patients suspected of having a viral infection from hepatitis C (HCV). Typically, such sera are subjected to RT-PCR to generate a DNA product that is specific to the HCV genome, which is subsequently analyzed by agarose gel electrophoresis. Felmlee et al. [66] applied CZE, coupled to LIF detection, to such a diagnostic problem. Analysis of 39 samples from patients by simultaneous CZE and agarose gel electrophoresis showed 100% correlation for the detection of a 308-bp fragment that is specific for HCV.

### 3.3. Polio virus

RT-PCR was adopted by Rossomando et al. [67] to produce DNA fragments that were specific for wild type polio virus (163 bp) and for three types of Sabin 1, 2 and 3 strains of vaccine polio virus (97, 71 and 53 bp, respectively). Good separation of such fragments was obtained by CZE in a proprietary Beckman sieving buffer, followed by detection and quantitation via LIF.

### 3.4. HIV-1 virus

Recently, a few groups have proposed the use of CZE with LIF detection for quantitating the PCR product from patients infected with the AIDS virus, after amplifying genomic DNA and cDNA by PCR and RT-PCR. Schwartz et al. [68] have adopted

separations in 0.5% HPMC-4000 and amplified a 115-bp fragment of the gag region of HIV-1. Lu et al. [69] have performed quantitative PCR of RT-PCR products with gag primers. These authors were able to screen sera with virion concentrations ranging from only 200 up to 500,000 viral particles per ml of serum. Williams et al. [70] also adopted quantitative RT-PCR and obtained from the HIV target gag-gene a 142-bp product DNA. Separations occurred in 3%T linear polyacrylamide and detection was via LIF.

## 4. Forensic medicine

The ability of PCR to make multiple copies from minute quantities of DNA extracted from blood, hair or semen has enabled new forensic approaches to human identification. Generally, DNA identification is based on differences in inherited genetic markers. Analytical techniques are employed following the PCR amplification to distinguish individuals based on these genetic differences, and CZE, due to its automation, speed and sensitivity, is now rapidly emerging as a unique tool in forensic medicine. A few examples will be given below.

### *4.1. Mitochondrial DNA (mtDNA)*

Samples of mtDNA can be extracted from human hair and/or blood samples found on a crime scene. Butler et al. [71] have amplified hypervariable areas of the control region of human mtDNA: HV1A (280 bp in length), HV1b (276 bp) and HV2 (416 bp). These fragments were well separated with high reproducibility and high sensitivity in a CZE system comprising 1% HEC and LIF detection via YO-PRO-1 dye intercalation. A similar mtDNA approach was proposed by Srinivasan et al. [72], who amplified 130–140 bp fragments and resolved then in 0.5% methyl cellulose (MC) with LIF detection via TOTO and YOYO intercalating dyes.

### *4.2. VNTRs at locus D1S80*

Genetic typing using PCR-amplified DNA is usually carried out on loci that contain a variable number of tandemly repeated sequences (VNTR). D1S80 is a marker located on human chromosome 1, locus 80, and it has a basic repeat unit of 16 bp. A standard mixture of alleles from D1S80 includes typically a whole series of fragments ranging in size from 403 to 1069 bp, with alleles being approximately 16 bp apart. McCord et al. [73] and Isenberg et al. [74] have shown the separation of a series of 22 such fragments in 0.5% HEC in a buffer containing ethidium bromide as the intercalator and cesium hydroxide as the mobility modulator. VNTRs from the same D1S80 locus were also analyzed by Srinivasan et al. [75], with the same dimeric dyes (TOTO and YOYO) proposed above [72].

### *4.3. VNTRs at locus HUMTH01*

This locus (TC-11) is located on chromosome 11 and is characterized by VNTRs with repeat units of 4 bp. McCord et al. [76] and Butler et al. [77] have amplified seven such HUMTH01 alleles, ranging in size from 183 to 207 bp, and have proposed separations in 1% HEC in the presence of a mixture of two intercalators, ethidium bromide and YO-PRO-1. A similar system was proposed also by Williams et al. [78], who additionally analyzed two other small tandem repeats (STR) systems: The mitochondrial dinucleotide repeat and the STRs ATT1–ATT5.

### *4.4. Amelogenin locus*

Another marker of marked forensic interest is the amelogenin locus. The typing of the amelogenin gene enables gender determination of the source of the DNA sample. This gene is carried on the sex chromosomes and the fragment of interest is six bp longer in the Y than in the X chromosome in humans. Isenberg et al. [74] have analyzed 18 and 22 repeat alleles belonging to males and 24 and 28 repeat alleles belonging to females in a sieving system composed of a mixture of two HECs: 0.28% larger HEC ($M_n$ ca. 150 000) and 0.3% smaller HEC ($M_n$ ca. 40 000). Additionally, they have proposed a multiplex assay comprising a mixture of fragments from the D1S80 locus and from the amelogenin locus.

## 5. Therapeutic DNAs

### *5.1. Antisense oligonucleotides*

Analysis and separation of antisense oligonucleotides (short segments of single stranded oligomers, typically 15–18-mers, complementary to a specific gene) has received much attention in recent times, due to their potential as therapeutic agents. A number of reports have recently appeared [79–86] describing the CZE separation of such oligonucleotides, all exploiting sieving liquid polymers in fairly high concentrations (up to 18% un-crosslinked polyacrylamide) and offering analysis times in a 20–30 min time frame. Gelfi et al. [87] recently adopted a slight variant of Cohen's buffer [83–85] (consisting in 267 m*M* Tris–borate buffer, pH 8.3, containing 2 m*M* EDTA, 6 *M* urea, 30% formamide and 18% acrylamide linear viscous polymer) and they have shown that indeed the separation of oligonucleotides in this medium was decidedly superior than in slab gel electrophoresis and in HPLC. However, in this ''syrupy'' solution, the analysis of 18-mer oligonucleotides required a total transit time of at least 30 min. In an attempt to ameliorate this analysis, Gelfi et al. [88] noted that CZE of the same compounds in isoelectric buffers (100 m*M* histidine) could be performed in as little as 4 to 5 min. In the latest report, Stoyanov et al. [89] extended the data of Gelfi et al. [88] and explored other types of buffers, such as isoelectric lysine. Unique results could be obtained by performing zone electrophoresis of oligonucleotides against a pre-established pH gradient, as is customary in isoelectric focusing techniques.

## 6. Double gradient DGGE

We have seen above that the DGGE technique, as performed in slab gels, is one of the most popular and most sensitive methodologies for the detection of DNA point mutations. It consists in co-amplifying the wild type (Wt) and presumptive mutant (M) DNAs so as to obtain, prior to analysis, a family of fragments; two hetero- and two homo-duplexes. Separation among the various members of the family will not occur when electrophoresis is performed in the absence of denaturants: The minute differences in the radius of gyration ($R_g$), due to the mismatch, between the two hetero- and homo-duplexes will only lead, at best, to band broadening. However, if the same set of duplexes is run against a gradient of denaturants (either chemical of thermal), each duplex will be intercepted, along the migration path, by an isoperichoric milieu in which the environmental conditions will match exactly the melting temperature of its lowest melting domain. This domain will suddenly be destabilized and a transition from an orderly helix to a partially unwound DNA molecule will take place, leading to strong retardation in the gel due to markedly increased frictional drag with the gel matrix and the solvent molecules. Due to small, but non-negligible, differences in $T_m$ values of the various duplexes, a spectrum of four bands is typically resolved, indicating the presence of a point mutation along a DNA filament. Small shifts in the $T_m$ of the corresponding melting domain may be due to transversions (AT→TA, GC→CG) or to mutations at the very end of cooperative melting domains, whereas switches of G:C pairs to A:T pairs and vice versa, small deletions or insertions, or multiple mutations, normally lead to larger shifts in $T_m$.

Structure denaturation by heat treatment or denaturing solvents is qualitatively similar, although helix-coil transitions of double-stranded nucleic acids, as elicited by temperature gradients, can be better described quantitatively by statistical thermodynamics. Notwithstanding the more cumbersome experimental set-up (often requiring the use of two thermostats for generating the temperature gradient, and of special precautions for avoiding unequal solvent evaporation from the gel slab), TGGE is also gaining in popularity and, at present, it is the only version we have been able to develop in a capillary format, under the name TGCE [24–26,42].

DGGE, as performed in the slab gel format, however, suffers from some inconveniences: Since the denaturant gradient slope and running times vary for every DNA region to be analyzed, different conditions have to experimentally found and optimized, greatly affecting the routine application of the methodology. In addition, since the running time needed for resolving the homo-duplexes often greatly exceeds that needed for separating the hetero-duplexes, the latter often produce smears and fuzzy bands, which are indistinguishable from the back-

ground fluorescence after ethidium bromide staining. It occurred to us that, if DGGE were to be performed in the presence of a second gradient, able to suppress band broadening until the very end of the analysis, one might obtain markedly improved patterns. A typical gradient that is able to minimize band broadening is a porosity gradient, as typically utilized, e.g., in native electrophoresis of complex protein mixtures or under denaturing conditions, in the presence of sodium dodecyl sulphate [90].

### *6.1. Analysis of p-53 point mutants*

We have recently reported the application of this double (porosity and denaturant, dubbed DG-DGGE) gradient for the detection of DNA point mutations, with improved banding patterns [91]. This marked improvement comes at minimal extra experimental burden, since the double gradient still requires the use of the same, single, two-vessel gradient mixer that is used for pouring the standard DGGE slab. This novel DG-DGGE technique was applied to the detection of a number of point mutations in the CFTR gene, with superior resolution and decidedly sharper zones [91]. We are presently applying this methodology to the screening of a vast number of point mutants in the p-53 gene, in collaboration with Dr. Zunino's group at the Istituto Nazionale dei Tumori, Milan, Italy. We offer here an example of the detection of four different mutations (Fig. 3): One can easily appreciate the sharpness of the homo- and hetero-duplex bands. The patterns obtained are similar to those routinely secured by isoelectric focusing in immobilized pH gradients, where band sharpening is a result of the interplay of two antagonistic forces; entropic ones, tending to spread the analyte zone into the surrounding space, and ''focusing'' forces, driven by the high voltage gradient, which tend to concentrate the sample in a very thin zone [92].

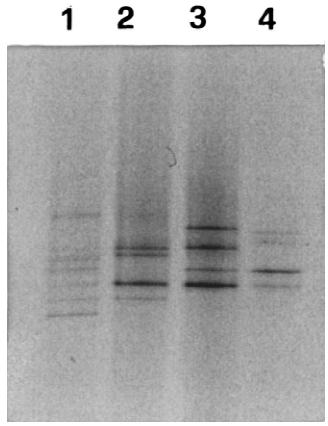


Fig. 3. DG-DGGE in slab gels for the resolution of p-53 point mutations. Gel: 6.5–12%T, 4%C polyacrylamide, 30–80% denaturant (formamide/urea) in TEA buffer (40 m*M* Tris, 20 m*M* NaOH, 1 m*M* EDTA, adjusted to pH 7.6 with acetic acid). Electrophoretic conditions: 75 V, overnight. Sample (from exon 6 of p-53 gene): 1=double heterozygous, A→G and T→C; 2= A→G transition; 3=G→T transversion at the splicing site; 4= C→T transition in codon 213. Post electrophoretic staining with ethidium bromide (C. Gelfi, P.G. Righetti, S.C. Righetti and C. Zunino, unpublished).

### *6.2. Conclusions*

CZE of DNA now seems to be a well standardized and reproducible technique, which has much to offer in the fields of genetic, medical and forensic analysis. Substantial improvements have recently been made in this field; among them (a) the advent of highly stable and hydrophilic inner coatings; (b) the production of short polyacrylamide chains of low viscosity for re-establishing constant initial conditions at the start of each run; (c) the development of temperature-programmed CZE for the detection of nearly 100% of point mutants. With the future introduction of multichannel instruments (e.g., batteries of 96 capillaries for the simultaneous analysis of 96 samples from microtiter plates), all coupled to fluorescence detection and to the possibility of activating thermal gradients, the CZE technique will become a competing methodology. Also, DGGE in gel slabs is now witnessing a revival with the introduction of double gradient techniques.

## 7. Abbreviations

| | |
|---|---|
| AAEE: | N-Acryloylaminoethoxyethanol |
| AAP: | N-Acryloylaminopropanol |
| ARMS: | Amplification refractory mutation system |
| bFGF: | Basic fibroblast growth factor |
| BMD: | Becker's muscular dystrophy |

| | |
|---|---|
| bp: | Base pair |
| CAIS | Complete androgen insensitivity syndrome |
| CF: | Cystic fibrosis |
| CFTR gene: | Cystic fibrosis transmembrane regulator gene |
| CMC: | Chemical mismatch cleavage |
| CZE: | Capillary zone electrophoresis |
| DGGE: | Denaturing gradient gel electrophoresis |
| DG-DGGE: | Double gradient, denaturing gradient gel electrophoresis |
| DMA: | N,N-Dimethylacrylamide |
| DMD: | Duchenne's muscular dystrophy |
| HA: | Heteroduplex analysis |
| HCV: | Hepatitis C virus |
| HEC: | Hydroxy ethyl cellulose |
| HIV: | Human immunodeficiency virus |
| HMC: | Hydroxymethylcellulose |
| HPMC: | Hydroxypropylmethylcellulose |
| LIF: | Laser-induced fluorescence |
| $M_n$: | Number-average molecular mass (for polymers) |
| $M_w$: | Weight-average molecular mass (for polymers) |
| PAA: | Polyacrylamide |
| PAGE: | Polyacrylamide gel electrophoresis |
| PAIS: | Partial androgen insensitivity syndrome |
| PCR: | Polymerase chain reaction |
| RFLP: | Restriction fragment length polymorphism |
| RT-PCR: | Reverse transcription polymerase chain reaction |
| SSCP: | Single stranded conformation polymorphism |
| STR: | Small tandem repeats |
| TGCE: | Thermal gradient capillary electrophoresis |
| TGGE: | Thermal gradient gel electrophoresis |
| Trisacryl: | N-Acryloyl-2-amino-2-hydroxymethyl-1,3 propanediol |
| VNTR: | Variable number of tandem repeats |

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