Chapter 6

Temperature and Denaturing Gradient Gel Electrophoresis

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6.1 INTRODUCTION

Temperature gradient gel electrophoresis (TGGE) and the related method denaturing-gradient gel electrophoresis (DGGE) are both based on the principle that the electrophoretic mobility of double-stranded DNA fragments is significantly reduced by their partial denaturation. Owing to the sequence dependence of the melting properties of DNA fragments, sequence variations can be detected. Although the sensitivity of TGGE and DGGE in detecting point mutations in genetic disorders and other settings has been reported to be close to 100%, these methods have never become as popular as other mutation detection methods such as SSCP (see Chapter 4), which may be related to the perception that it is difficult to design adequate PCR primers and set up the assays. In this chapter, the basic principles of TGGE/DGGE will be discussed and procedures for setting up assays will be described, including how to design and test PCR primers suitable for TGGE/DGGE analysis. Furthermore, studies on the sensitivity of TGGE/DGGE for mutation analysis of genetic disorders will be reviewed and an overview of variations on the basic TGGE/DGGE method will be provided. TGGE and DGGE are robust and highly sensitive methods for mutation screening of genetic disorders that have many advantages, which counterbalance the extra effort required in establishing the method.

6.2 THE THEORY OF TEMPERATURE-GRADIENT GEL ELECTROPHORESIS

6.2.1 Melting Behavior of Short Double-Stranded DNA Fragments

Myers and coworkers (1985b) originally developed a method of separating DNA fragments differing by single nucleotide substitutions in denaturing gradient gels. The method was based on the notion that the denaturation (melting) of DNA fragments can be regarded as an equilibrium for each base pair (bp) between two distinct states: (1) double helical, and (2) a more random state in which bases are neither paired nor stacked on adjacent bases in any orderly way (Myers et al., 1987). The change from the first to the second state is caused by increasing temperature or increasing concentration of denaturing agents.

In the case of single-nucleotide substitutions, the replacement of an A:T bp (two hydrogen bonds) by a G:C pair (three hydrogen bonds) will generally be expected to increase the temperature at which the corresponding DNA sequence melts. The context of the nucleotide substitution also plays a role, and substitutions of A:T by T:A pairs, or G:C by C:G pairs, can also affect the temperature at which a DNA sequence dissociates.

Furthermore, a DNA fragment dissociates in a stepwise fashion as the temperature is gradually increased. Dissociation occurs nearly simultaneously in distinct, approximately 50 to 300 nucleotide long regions, termed “melting domains”. All nucleotides in a given melting domain dissociate in an all-or-nothing manner within a narrow temperature interval.

The melting temperature ($T_m$) indicates the temperature at which 50% of the individual molecules are dissociated in the given melting domain, and 50% are double helical. As indicated above, the $T_m$ is strongly dependent on the individual DNA sequence and can be significantly altered by small changes in the DNA sequence including single-nucleotide substitutions.

6.2.2 Electrophoretic Mobility and the Melting State of DNA Fragments

TGGE is based on detecting differences in the electrophoretic mobility between molecules that may differ only at a single position. DNA fragments produced by
the polymerase chain reaction (PCR) are subjected to electrophoresis through a linearly increasing gradient of temperature (or concentration gradient of denaturing agents such as urea and formamide for DGGE). Nucleotide substitutions and other small changes in the DNA sequence are associated with additional bands following TGGE.

The electrophoretic mobility of DNA fragments differs according to whether the fragment is completely double helical, if one or more melting domains has dissociated, or if complete dissociation to two single-stranded molecules has occurred. Each of these states can be visualized using a perpendicular TGGE experiment, as will be discussed further below (see section 6.3.2).

The electrophoretic mobility of a double helical (non-denatured) DNA fragment is not significantly altered by single-nucleotide substitutions within it, but is primarily dependent on the length and perhaps the curvature of the fragment (Haran et al., 1994). Therefore, assuming that PCR products contain a mixture of two DNA fragments that differ at a single position, as would be the case for a heterozygous point mutation, both fragments will initially progress through the gel at the same speed.

When the molecules reach that point in the gel where the temperature equals their \( T_m \), the molecules will experience a decrease in mobility owing to a transition from a completely duplex (double helical) conformation to a partially denatured one. Dissociation of the first or first few melting domains generally results in a dramatic reduction in the mobility of the DNA fragment, because the fragment takes on a complex, branched conformation.

Due to the strong sequence dependence of the melting temperature, branching (dissociation) and consequent retardation of electrophoretic mobility occurs at different levels of the temperature gradient associated with bands at different positions in the gel (Myers et al., 1987). In addition to the two homoduplex molecules (wt/wt and mt/mt), two different heteroduplex molecules (wt/mt and mt/wt) can be formed by dissociating and reannealing DNA fragments containing a heterogeneous mutation prior to performing TGGE (Fig. 6.1). In practice, it is also possible to perform 40 cycles of PCR; the activity of the Taq polymerase is exhausted in the final cycles of PCR, such that heteroduplexes are formed as efficiently as if one performed denaturation and reannealing following PCR. Heteroduplex fragments then contain unpaired bases or “bulges” in the otherwise double helical DNA, resulting in a significant reduction in the \( T_m \) of the affected melting domain (Ke and Wartell, 1995). The melting temperatures of the two heteroduplex molecules are generally different from one another, so that each heteroduplex is separately visible in the gel. A heterozygous point mutation will thus be visualized by the appearance of four bands: a band representing the normal allele (homoduplex), a band representing the mutant homoduplex that will lie above or underneath the wild-type homoduplex band, depending on the effect of the mutation on the \( T_m \), and two heteroduplex bands that are always above the homoduplex bands (Fig. 6.2; Myers et al., 1987).

In the discussion above, a significant issue is that mutations are detectable only in the melting domain(s) with the lowest melting temperature. If, however, a DNA molecule contains several melting domains with different melting temperatures, it is generally not possible to visualize mutations located elsewhere than in the melting domain with the lowest \( T_m \). Once the DNA fragments reach the temperature at which the first melting domain dissociates, the mobility of the fragment is greatly reduced so that it may not reach temperatures relevant for the higher \( T_m \) domains under the conditions of the experiment. Also, dissociation of the highest \( T_m \) domain results in complete dissociation of the DNA fragment into two single-stranded DNA molecules. Single-stranded DNA, like completely double helical DNA, does not demonstrate differences in electrophoretic mobility, owing to small sequence changes, and hence there is no possibility of distinguishing two sequences once complete dissociation has occurred.

6.2.3 Mutations are only Detectable in the Lowest Melting Domain(s)

The consequence of these observations is that only mutations in the lowest \( T_m \) domain can be reliably detected by TGGE or DGGE (Myers et al., 1987).

6.2.4 GC- and Psoralen Clamps Extend the Usefulness of TGGE

Myers and coworkers (1985a) presented an extension of the original DGGE protocol that allowed mutations in every region of the DNA fragment under analysis to be detected.
These researchers attached a 135 bp, GC-rich sequence, known as “GC-clamp”, to the β-globin promoter region in which mutations were being sought. The β-globin promoter region was found to contain two melting domains; without the GC-clamp, only mutations in the domain with the lower $T_m$ could be visualized in the gel. Owing to its high GC content, the GC-clamp has a significantly higher melting temperature than most naturally occurring sequences. The attachment of the GC-clamp was found to significantly alter the melting properties of the β-globin sequence and mutations in the entire β-globin sequence could be experimentally detected (Myers et al., 1985a). By adding a 40nt G + C-rich sequence to one of the two PCR primers, a GC-clamp can be conveniently added to any DNA fragment produced by PCR (Sheffield et al., 1989). It is also possible to use a universal GC-clamp that is incorporated into amplified DNA fragments during PCR, thereby avoiding the expense of synthesizing long primers (Top, 1992).

Psoralen-modified PCR primers are an alternative to GC-clamps. One of the two PCR primers is 5′ modified by 5-(ω-hexyloxy)-psoralen. The 5′ terminus of the primer should have two adenosine residues; if the natural sequence does not have AA, this sequence should be appended to the specific DNA sequence of the primer. Psoralens are bifunctional photoreagents that can form covalent bonds with pyrimidine bases (especially thymidine). If intercalated at 5′-TpT in double helical DNA (this will be the complementary sequence of the 3′ terminus of the other strand following PCR), psoralen forms a covalent bond with thymidine after photoinduction (Costes et al., 1993b). Photoinduction can be performed by exposing the PCR products to a source of UV light (365) for 5 to 15 minutes, which can be conveniently done in the original PCR tubes or 96-well plate.

In general, psoralen clamping provides comparable results to GC clamping, except that cross-linking of the PCR fragments is only approximately 85% efficient, so that one observes single-stranded, denatured DNA fragments running below the main bands in the TGGE. Psoralen clamping is sometimes preferred over GC-clamping because the PCR is often easier to optimize, and bipolar clamping is possible if necessary (see below). Psoralen modification of primers is available from many commercial oligonucleotide sources.

6.3 THE PRACTICE OF TEMPERATURE GRADIENT GEL ELECTROPHORESIS

Detailed protocols for TGGE and DGGE are available elsewhere (Kang et al., 1995; Murdaugh and Lerman, 1996). In the following paragraphs, the most important issues concerning how to set up TGGE or DGGE assays successfully are discussed, including especially the issues related to primer design and optimization procedures. Several points that apply only to DGGE are discussed in section 6.4.

6.3.1 Primer Design for TGGE/DGGE

One of the first and most widely used computer programs to design primers for TGGE was the Melt87 package by Lerman and Silverstein (1987). An updated version of this program (Melt94) is available at http://web.mit.edu/osp/www/melt.html. The Melt87 program calculates the $T_m$ for each bp in the DNA fragment, i.e. the temperature at which 50% of the individual molecules are double helical and 50% of the molecules are in a fully disordered, melted state. The results of such a calculation are termed “melting map” (Fig. 6.3). One notices that DNA fragments are typically divided into distinct melting domains of about 50 to 300bp in length, in which all base pairs have nearly identical $T_m$. The melting map demonstrates the lowest melting domain in the DNA fragment; as mentioned above, only mutations in this region will be visible by TGGE analysis.

A further useful program in the Melt87 package is SQHTX. This program calculates the expected displacement in the gradient for a single-nucleotide mismatch (as would be the case for a heteroduplex molecule with a single-nucleotide
substitution) at every position in the fragment. This analysis provides the clearest indication of the position in the fragment, where mutations will be detectable by TGGE analysis (Lerman and Silverstein, 1987). Figure 6.4 provides an example of a displacement map calculated with SQHTX.

The Melt87 programs are DOS-based and difficult to use for those with little experience with DOS and menu-based programs. Melt87 has no graphic capabilities of its own, and users need to process its output with a graphics program of their choice. For this reason, several freely available and proprietary programs have become available, which are significantly easier to use (Table 6.1).

![Figure 6.3 Melting map](image1.png)

**FIGURE 6.3** Melting map. This graphic represents a fragment from exon 14 of the NF1 gene and was produced using TGGE-Star. Each tick on the x-axis represents a base pair. The base pairs are numbered from 1 to 195. The y-axis shows the temperature where the probability for a bp to be melted has the value 0.95, 0.75, 0.5, 0.25, and 0.05, respectively. The 5' terminus of the fragment corresponds to a GC-clamp. Additionally, one can distinguish two further melting domains: from the 5' terminus to the 50th bp and from the 50th bp to the 3' terminus. The difference between these two melting domains is small and the sensitivity of TGGE is not disturbed. If the difference between these two plateaus in the curve were higher, both regions would need to be tested in two different PCR-TGGE steps. Mutations were detected in both regions of this fragment: three asterisks above the x-axis mark positions of mutations detected with this assay.

![Figure 6.4 Displacement map](image2.png)

**FIGURE 6.4** Displacement maps calculating using the program SQHTX, and graphic created with TGGE-Star. In the case of a heterozygous mutation, two heteroduplex bands occur. Heteroduplexes do not migrate as far as the wild-type fragments because they “melt” at lower temperatures. The distance of heteroduplex bands and wild-type bands depends on the electrophoretic duration (x-axis) and the base position (y-axis). A mutation can only be detected, when the displacement is higher than the resolution of the gel. The color codes indicate different electrophoretic times, and the width of each band of color indicates the expected displacement (in arbitrary units) in the gel for a point mutation at the corresponding position in the sequence. Note: The e-book for this title, including full-color images, is available for purchase at www.elsevierdirect.com.

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<td>Server-based implementation of Poland’s algorithm (Steger, 1994)</td>
<td><a href="http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html">http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html</a></td>
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<tr>
<td>MELTingeny</td>
<td>A commercial, Java-based GUI program with flexible routines for designing DGGE/TGGE primers</td>
<td><a href="http://www.ingeny.com">http://www.ingeny.com</a></td>
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<td>WinMelt, MacMelt</td>
<td>Commercial GUI programs for melting profile analysis</td>
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the 5’ or 3’ PCR primer or both (see below for discussion of bipolar clamping). Programs such as TGGE-Star and MELTingeny facilitate this process by allowing users to easily shift primer positions and recalculate the melting maps. It should be mentioned that a 40-nucleotide GC clamp can be “substituted” for a psoralen clamp in the computer analysis.

6.3.2 Perpendicular TGGE for the Determination of the $T_m$

In most cases in which TGGE is used for mutation analysis, parallel electrophoresis with simultaneous analysis of multiple samples will be performed. For each such assay, the optimal temperature gradient and run time must be determined experimentally. The procedures used for this purpose are described in this and the following paragraph.

The optimization process begins with a perpendicular TGGE experiment, in which electrophoresis is performed perpendicularly to the temperature gradient (Fig. 6.5). Perpendicular TGGE is used to verify the reversible melting behavior of the DNA fragment and to determine its $T_m$ under the experimental conditions. Perpendicular TGGE is run with a gradient of 20°C–60°C, which will be adequate for the vast majority of PCR fragments. Electrophoresis is initially performed at room temperature for 10–15 minutes to run the sample into the gel. Then, electrophoresis is stopped while a temperature gradient of 20°C–60°C is established, after which electrophoresis should be continued for 90–120 minutes. Figure 6.5 demonstrates the use of this analysis to determine the $T_m$ of the DNA fragment being analyzed.

6.3.3 Travel Schedule Experiments

Up to three novel bands are observed upon TGGE/DGGE analysis of a heterozygous mutation or polymorphism. The separation will begin to become apparent when the heteroduplex molecules have reached their $T_m$, as their mobility will be retarded by partial denaturation. Separation of the homoduplex molecules will occur in a region of the gradient surrounding the $T_m$ of the lowest melting domain of the DNA fragment. Therefore, TGGE assays are set up to avoid a long running time before the samples reach the effective range of separation. One should choose the temperature gradient such that the effective range of separation is approximately in the middle or somewhat above the middle of the gel, and that the upper and lower temperature ranges are separated by about 15°C from the $T_m$ of the DNA fragment.

Once an appropriate temperature gradient has been chosen, the optimal running time can be determined by a travel schedule experiment, which is a parallel TGGE experiment in which samples are applied every 30 minutes for 3 hours (or longer), such that the last sample to be loaded has run 30 minutes, and the first sample 3 hours. Usually, one will see a reduction on electrophoretic mobility of samples after a certain period of time (generally 60–90 minutes if the temperature gradient was chosen correctly). Samples often do not continue to wander in the gel with any significant velocity once their melting temperature has been reached. These gels are generally run for about 30 minutes longer than the time determined in this manner (Fig. 6.6). Different choices of the range and starting point of the temperature gradient affect both the range in the gel at which mutations will be visible as well as the optimal running time (Fig. 6.7).

**FIGURE 6.5** A. Schematic drawing of a perpendicular TGGE/DGGE gel. A temperature gradient from t1 (e.g. 20°C) to t2 (e.g. 60°C) is established perpendicularly to the direction of electrophoresis (indicated by “−” and “+”). Fragments at lower temperatures remain completely double helical and have a relatively high electrophoretic mobility (“a”). Once the melting temperature of the lowest-temperature melting domain is reached, partial denaturation of the DNA fragment (“b”) causes a significant reduction of electrophoretic mobility. The temperature at which 50% of individual molecules are melted is denoted as the “melting temperature” ($T_m$), and is indicated by the arrow in the figure (“b’”). A reversible denaturation step is observed as a continuous transition (curve). Once the temperature of the highest-melting domain is reached, irreversible melting occurs, causing a discontinuous transition in the melting curve (“c”). B. Perpendicular TGGE gel. In this example, a PCR fragment corresponding to NF1 gene exon 14 was analyzed. PCR product was applied and run into the gel at 10°C for 15 minutes. Then, a temperature gradient from 20°C to 60°C was established perpendicularly to the direction of electrophoresis, which was then performed for an additional 60 minutes. One observes a high electrophoretic mobility in portions of the gel with temperatures below the $T_m$ of the fragment. The gradual decrease in mobility around the middle of the gel indicates reversible melting of the lowest-temperature melting domain. In portions of the gel with temperatures above the $T_m$ of the fragment, partial denaturation of the fragment leads to a significantly reduced electrophoretic mobility. The arrow at the midway point of the curve indicates the $T_m$ of the fragment under the experimental conditions (approximately 39°C).
6.3.4 Bipolar Clamping

Occasionally, TGGE analysis will result in fuzzy bands that are difficult to evaluate, despite apparently adequate melting behavior, as predicted by Mehl94 or other programs. Bipolar clamping of PCR products, by means of attaching a psoralen clamp to each of the two PCR primers rather than just one, is an effective method to improve melting characteristics of PCR fragments that are otherwise not amenable to TGGE/DGGE analysis (Gille et al., 1998). Bipolar clamping is a simple procedure that can significantly improve results of TGGE analysis in cases where analysis with only one clamp has yielded suboptimal results. Programs such as TGGE-Star (Gille and Gille, 2002) offer the possibility of computer analysis with two clamps, and may suggest the use of bipolar clamping for amplicons whose predicted melting properties are otherwise not satisfactory.

6.4 Denaturing Gradient Gel Electrophoresis (DGGE)

The theory of DGGE/TGGE is described in detail in the first part of this chapter. Parallel DGGE is a form of polyacrylamide gel electrophoresis in which a double-stranded DNA fragment migrates into a gradient of linearly increasing denaturing conditions. The denaturing gradient is functionally equivalent to the temperature gradient of TGGE. The denaturants used are heat (a constant temperature of generally 60°C) and a fixed ratio of formamide (ranging from 0 to 40%) and urea (ranging from 0 to 7M). The temperature of 60°C was empirically chosen to exceed the melting temperature of an AT-rich DNA fragment in the absence of a denaturant. For extremely GC-rich DNA
sequences higher temperatures (e.g. 75°C) can be used.
To achieve a uniform temperature distribution the electrophoresis unit is attached to a circulating water bath.

### 6.4.1 Optimization of Gel Running Conditions

The computer programs (e.g. Melt94) described above reduce the number of preliminary experiments required for optimization of the gel running conditions. However, it is still necessary to run some preliminary gels to determine the optimal electrophoresis conditions and running times and to confirm that the optimal denaturing gradient has been chosen. The aim of these travel schedule gels is to have well separated bands (normal and mutation positive control are simultaneously loaded on the gels), which are “focused” by the gradient. PCR products with two low-melting domains require different gel conditions for the analysis of each domain.

The choice of the denaturant concentration range can be determined as follows. The differences in gradient depth (the displacement) between a fragment and the same fragment with a change at a specified bp are calculated by the program SQHTX (Lerman and Silverstein, 1987) as described in section 6.3.1. SQHTX calculates the displacement as the difference in temperature at which the wild-type homoduplex and the heteroduplex molecules partially melt (Fig. 6.4). To convert between the temperature values and the denaturant concentration, a difference of 1°C is converted to a difference of 3% denaturant concentration (approximately equivalent to 1 cm distance within a 20% urea gradient gel). An experimental determination of gradient behavior can be achieved by perpendicular gel electrophoresis. Data from the perpendicular gels help to estimate the denaturant concentration range to use in parallel gel electrophoresis. For parallel gels, the gradient should be initially chosen with a 25% to 30% difference in denaturant concentration centered around the melting temperature of the domain (Myers et al., 1987). Once optimized gel running conditions have been established, the method can be used for mutation screening.

### 6.5 THE USE OF TGGE/DGGE FOR MUTATION DETECTION

TGGE and DGGE have been used to investigate a large number of disease genes, some of which are listed in the following sections. Due to the relative ease of detecting heterozygous mutations owing to the occurrence of up to three novel bands, TGGE and DGGE have been particularly useful for disorders characterized by heterozygous mutations or frequent de novo mutations (reviewed in Fodde and Losekoot, 1994). In light of the effort involved in designing primers and optimizing conditions, TGGE or DGGE is generally reserved for situations when large numbers of samples are to be screened for mutations.

Most mutation screening protocols involve the simultaneous analysis of 24 or more samples on one parallel TGGE/DGGE. In general, altered band patterns are easy to spot. The classical appearance of heterozygous mutations (Figs 6.2 and 6.8) is due to the appearance of three additional bands. With some mutations, only one or two additional bands are seen. In the authors’ experience, the specificity of TGGE/DGGE is exquisitely high. In other words, a false-positive four-band pattern has never been observed.

### 6.6 DETECTION RATE AND SENSITIVITY

By using DGGE, Myers and coworkers (1985b) detected an estimated 40% of the sequence variants in a DNA fragment up to 500 bp in their initial study. The use of GC-clamps, psoralen clamps, or bipolar clamping, which aids the formation of uniform low melting domains, significantly improved the detection rate of TGGE/DGGE, which in many cases approaches nearly 100%.

The sensitivity of TGGE/DGGE for detecting known mutations is generally reported to be nearly 100%, generally performing as well or better than other mutation detection methods (Abrams et al., 1990; Ferec et al., 1992; Gelfi et al., 1997; Gejman et al., 1998; Tchernitchko et al., 1999; Zschocke et al., 2000; Breton et al., 2003). In one study with a panel of known mutations, DGGE detected 201 of 201 known mutations in the CFTR gene (Macek et al., 1997). The reasons for lower reported detection rates of unknown mutations in some studies has been speculated to be due to genetic heterogeneity (Ferec et al., 1999), clinical overdiagnosis (Katzke et al., 2002) or location of mutations in intronic or promoter regions that were not included in the screening program. Optimization of the TGGE/DGGE assay conditions and primers, perhaps including the use of bipolar clamping (Gille and Gille, 2002), may increase sensitivity. In summary, the sensitivity of TGGE/DGGE, when properly used, is close to 100%.

TGGE/DGGE has also been shown to be very sensitive in the detection of mutations in situations where the
mutation sequence is present in proportions less than 50% (as is generally the case when heterozygous mutations are sought in genomic DNA). This has proved useful in detection of heteroplasmy in mitochondrial disorders with heteroplastic proportions as low as 1% (Tully et al., 2000), as well as in testing for residual disease in cancer (Ahnhudt et al., 2001; Alkan et al., 2001).

6.7 RELATED TECHNIQUES AND VARIANTS

A wide range of improvements and further developments of the principles underlying DGGE and TGGE have appeared in the last decade, the most important of which are briefly summarized below.

In **Broad range DGGE**, a single gel and a single set of conditions are used to screen all the exons of one gene (Guldberg and Guttler, 1994; Hayes et al., 1999).

In **Multiplex DGGE**, several exons are simultaneously analyzed in one DGGE gel (Costes et al., 1993a).

In **Genomic DGGE** (gDGGE), genomic DNA is digested with a restriction enzyme, electrophoresed by DGGE, transferred to nylon membrane and hybridized to a unique DNA probe (Borresen et al., 1988).

In **Constant DGGE** (cDGGE), gels contain constant concentrations of denaturants. This allows an increased resolution of mutant fragments since they will constantly migrate with a different electrophoretic mobility through the whole length of the gel (Hovig et al., 1991).

In **Constant denaturant capillary electrophoresis** (CDCE), DNA migrates through a 30 cm quartz capillary of 75 μm inner diameter, filled with a viscous polyacrylamide solution. A 10 cm part of the capillary, prior to the detector, is heated to a temperature permitting partial melting (see also previous chapter). Usually, the DNA is fluorescently labeled and detected by laser-induced fluorescence (Khrapko et al., 1994). Separation of DNA fragments is achieved by the differential velocity of partly melted DNA in a medium with uniform denaturant concentration. Chip-based variants of temperature gradient capillary electrophoresis have recently been developed (Zhang et al., 2007).

In **Temporal temperature gradient gel electrophoresis** (TTGE), a constant concentration of urea or formamide is used as in cDGGE but the temperature during the run is gradually increased (Yoshino et al., 1991; Wiese et al., 1995). The denaturant concentration (usually 6–8% urea) used in TTGE can be determined either from the theoretical melting curve or experimentally from a perpendicularly DGGE.

In **Microtemperature-gradient gel electrophoresis** (μTTGE), a minimized gel (20 × 20 × 0.5 mm) leads to the reduction of the amount of DNA required and to shorter running times (approximately 12 min at 100 V, 10 mA). The method was used in microbial ecology and epidemiology (Biyani and Nishigaki, 2001; Tominaga, 2007).

In **Double-gradient, denaturing gradient gel electrophoresis** (DG-DGGE), in addition to the chemical denaturing gradient (formamide and urea) a second sieving gradient (e.g. 6–12% polyacrylamide gradient) is used (Cremonesi et al., 1997).

The **Two-dimensional DNA fingerprinting/two-dimensional gene scanning** (TDGS, 2D-DNA typing), combines size separation of DNA fragments in the first dimension with their sequence-specific separation through DGGE in the second dimension (see also next chapter).

**Denaturing HPLC** (dHPLC) uses an ion-pair chromatography separation principle, combined with a precise control of the column temperature and optimized mobile phase gradient for separation of mutant DNA molecules (reviewed in Xiao and Oefner, 2001).

In **Cycling gradient capillary electrophoresis** (CGCE), DNA sequence variants are detected based on their differential migration in a polymer-filled capillary system. A cycling (oscillating) temporal temperature gradient is applied. This improvement enables utilization of a multiple injection technique, in which multiple samples are injected into the same capillary (or set of capillaries) separated by predefined time intervals of partial electrophoresis. A 96-capillary system is able to screen over 15,000 samples in 24 h (Minarik et al., 2003).

6.8 TECHNICAL EQUIPMENT FOR TGGE/DGGE

In general, for DGGE, pre-existing vertical electrophoresis equipment with buffer-tank and combined heater/stirrer thermostat can be adapted. For TGGE, special equipment to achieve a constant temperature gradient is necessary.

The **Biometra TGGE** (Goettingen, Germany, http://www.biometra.de) system uses a temperature block powered by Peltier technology, which enables a strictly linear gradient that may allow more reproducible conditions than with conventional chemical gradients or temperature gradients using water baths. The Biometra TGGE system is available in two formats: a TGGE “mini” system operates small gels and is therefore suitable for fast, serial experiments and a TGGE maxi system provides a large separation distance and allows high parallel sample throughput.

The **DCode mutation detection system** (Bio-Rad Laboratories, Hercules USA, http://www.biorad.com) can be used to screen mutations by DGGE, TGGE, CDGE, TTGE, and by other techniques. The system performs TTGE by controlling the buffer temperature during the electrophoresis run. A temperature control module regulates the rate of temperature increase in a uniform and linear fashion.

**Sooner Scientific** (Garvin, USA, http://www.soonersci.com) offers five different sized DGGE Systems variants (for 2, 4 or 8 smaller gels or one large gel).

6.9 Applications of TGGE/DGGE and Related Methods

TGGE/DGGE has been applied in an increasing number of studies. A recent search in PubMed database found over 2,200 citations (assessed in July 2008). The following applications have been described:

- Screening for polymorphisms in human genes: e.g. COLIA2 gene (Borresen et al., 1988), SERPINA1 (alpha-1-antitrypsin; Hayes, 2003), HBG1/HBG2 (human gamma-globin genes, Patrinos et al., 1998, 2001; Fig. 6.9).
- Mutation detection in human genes: e.g. p53 (Pignon et al., 1994), FBN1 (Tiecke et al., 2001; Katzke et al., 2002; Robinson et al., 2002), NFI (Peters et al., 1999; Fahseld et al., 2000), Dystrophin gene (Hofstra et al., 2004), HBD (human beta-globin, Papadakis et al., 1997) and HBB (beta-globin) genes (Losekoot et al., 1990; Fig. 6.9), multiple endocrine neoplasia type 1 (Balogh et al., 2004), and Y-chromosomal microdeletions (Bienvenu et al., 2003).
- Mutation and single nucleotide polymorphism (SNP) detection with chip-based temperature gradient capillary electrophoresis (Zhang et al., 2007).
- Mutation and polymorphism detection in mitochondrial DNA (Hanekamp et al., 1996; Chen et al., 1999).
- Analysis in microbial ecology, determination of biodiversity of bacterial populations in soil, fresh or salt water (Muyzer and Smalla, 1998; van Elsas et al., 2002), in rumen microbial populations (Deng et al., 2008), and detection of microbes in food (Ercolini, 2004).
- Genome profiling and provisional microbial species identification on the basis of random PCR and TGGE (Watanabe et al., 2002).
- Determining of bio-diversity in fecal or intestinal microflora (Tannock, 2002), and in endodontic infections (Siqueira et al., 2005).
- HLA typing (Uhrberg et al., 1994; Knapp, 2005).
- Analysis of proteins and antibody binding (Riesner et al., 1991; Arakawa et al., 1993).
- Clonality analysis of T-cell or T-cell receptors (Plonquet et al., 2002; Lukowsky, 2003).
- Mutation detection and detection of variation between genomes of viral strains (Lu et al., 2002; Motta et al., 2002).
- Analysis of bio-diversity and polymorphisms in plants (Gomes et al., 2003; Nikolcheva et al., 2003).
- Examination of the fidelity of DNA polymerases (Keohavong and Thilly, 1989).
- High-throughput discovery of SNPs and other genetic polymorphisms (Hsia et al., 2005; Maher et al., 2006).

6.10 Conclusions

TGGE/DGGE and related methods provide a very high sensitivity and are relatively easy and cheap to perform once the assays have been designed and optimized. The main advantages consist in the high detection rate and specificity and improved heterozygote detection. The methodology is simple, non-radioactive, and relatively non-toxic. The disadvantages of TGGE and DGGE include mainly the limitation of PCR fragment length to about 500 nucleotides, the difficulties of analyzing GC-rich fragments, and the need for computer analysis of potential PCR fragments (which on the other hand can save time and money by eliminating the use of inadequate primers). However, once primers and conditions have been chosen, TGGE/DGGE is a robust and easy to perform mutation screening method. It is particularly well suited for the detection of known and unknown mutations in large genes, where high sensitivity is required and when large numbers of samples are to be tested.

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