Temporal Temperature Gradient Electrophoresis — A Powerful Technique to Screen Mutation

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Introduction

As the number of diseases and disorders linked to gene mutations grow, the need for scanning single-base mutations becomes ever more vital to extending understanding of disease-risk predictor mutations and their outcome. Since most mutations are single-base changes, a number of methods that are faster and more economical than DNA sequencing have been developed. Single-stranded conformation polymorphism (SSCP) (Orita et al. 1989), denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman 1979), constant denaturing gel electrophoresis (CDGE) (Hovig et al. 1991), temperature gradient gel electrophoresis (TGGE) (Rosenbaum and Riesner 1987), heteroduplex analysis (HA) (Nagamine et al. 1989), protein truncation test (PTT) (Roest et al. 1993), and enzymatic/chemical cleavage analysis are the major detection methods used to screen mutations.

The DCode™ universal mutation detection system (Figure 1) can be used to screen mutations by any of the techniques mentioned above and by a new technique called temporal temperature gradient electrophoresis (TTGE).

Temporal temperature gradients previously described by Yoshino et al. (1991) and Wiese et al. (1995) have been used for electrophoretic separations where the spatial temperature gradient is replaced with a temporal temperature gradient.

Fig. 1. The DCode system is a flexible system that can be used for any combination of mutation screening techniques. At the center of the system is the temperature control module, which includes a microprocessor-controlled heater, a buffer recirculating pump, and a stirrer. For techniques requiring temperature control, the gels are immersed in the buffer and the temperature is regulated between 5° and 70°C. Temperatures below ambient can be achieved by using the cooling tank in conjunction with an external laboratory chiller.

In TTGE, the denaturing environment is formed by a constant concentration of denaturant in the gel in combination with the temporal temperature gradient. During electrophoresis, the temperature is increased gradually and uniformly. This results in a linear temperature gradient over the course of the run (Figure 2). Electrophoretic mobility is decreased as DNA denaturation begins. Mutant and wild-type molecules have different melting behavior and thus begin to denature at different points in time resulting in a separation on the gel.

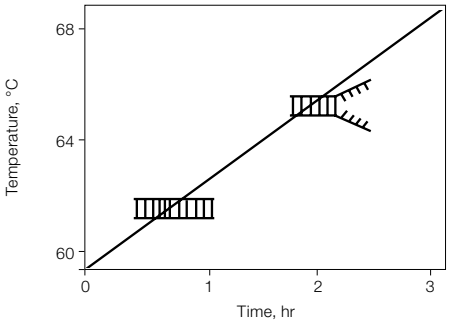

Fig. 2. TTGE is based on the same principle as DGGE, without the chemical gradient. The denaturing environment is formed by a standard urea gel and a gradual temperature ramp over the course of the run. As the temperature increases, the double-stranded DNA will become partially melted.

The DCode 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. This temperature increase, or ramp rate, can be controlled from 0.1–26°C per hour. The data presented describe the principle of TTGE and optimization parameters with examples.

Methods

Mutant and wild-type genomic DNA were isolated from cell cultures. The samples were amplified for exon 8 of the *p53* gene according to Borresen et al. (1991), using a 20-mer primer (5'-ATCCTGAGTAGTGGTAATCT- 3') and a 60-mer primer (5'-GCGGGCGGCGCGGGGCGCGGGCAGGGCGGCG-GGGGCGGGCTACCTCGCTTAGTGCTCCCT- 3'), which contains a 40-base pair (bp) GC clamp. Amplifications were carried out using 100 ng DNA template in 1x PCR buffer (PerkinElmer, Inc.), 0.2 mM each dNTP, Taq DNA polymerase

(PerkinElmer), 20-mer primer (25 pmol), and 60-mer primer (25 pmol). Conditions for PCR were: 94°C for 2 min, 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec; final extension at 72°C for 10 min. The reaction products were checked on a Ready Gel® 10% TBE gel (Bio-Rad Laboratories, Inc.) for size homogeneity of 189 bp.

Mutant and wild-type β-thalassemia DNA were isolated from blood. Amplifications were carried out using 100 ng DNA template in 1x PCR buffer, 0.2 mM each dNTP, Taq DNA polymerase, 25 pmol of a 21-mer primer (5'-CGTGGATGAA-GTTGGTGTGA- 3') and 25 pmol of a 60-mer primer (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG-CCCC-CGCCCGCCCATAACAGCATCAGGAGT- 3'). Conditions for PCR were: 94°C for 2 min, 35 cycles of 94°C for 1 min, 63°C for 1 min, 72°C for 1 min; final extension at 72°C for 10 min. The reaction products were checked on a Ready Gel 10% TBE gel for size homogeneity of 281 bp.

TTGE gels (10 x 8 x 0.1 cm) contained polyacrylamide/bis (37.5:1) in varying concentrations of TAE buffer (1x: 40 mM Tris acetate, 1 mM EDTA, pH 8.0) and varying concentrations of urea. Samples were electrophoresed on the DCode system (Bio-Rad) as described in the figure captions. After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide in TAE running buffer for 5 min, destained for 20 min, and then photographed on an ultraviolet (UV) transilluminator.

Results and Discussion

Temperature Range and Denaturant Parameters

There are two approaches for determining denaturant concentration required in TTGE. One is based on results of a perpendicular DGGE gel. For example, Borresen et al. (1997) cast the TTGE gels with a denaturant concentration approximately 10 units lower than the denaturant concentration at the steepest part of the S-shaped curve on the perpendicular DGGE gel. Thus, a 50% denaturant concentration at the steepest part of the S-shaped curve on the perpendicular DGGE requires a TTGE gel with 40% denaturant.

Another approach uses the theoretical melting behavior of the DNA sequence as a means to determine the temperature range and denaturant concentration. In this method, the first step is to ascertain the melting profile of the sequence of interest. Bio-Rad offers Mac- and PC-based programs (MacMelt™ and WinMelt™ software) that calculate and graph the theoretical melting profiles of a DNA sequence. The software is based on the melting algorithm from the original Melt87 software developed by Lerman and Silverstein (1987). The temperature range for the gradient can be calculated from the melting profile graph by first determining the lowest and the highest non-GC-clamped melting temperature of the DNA sequence. From the calculated low and high temperatures, the theoretical melting temperatures can be lowered by adding urea to the gel. A denaturing urea gel will lower the theoretical melting temperature of DNA by 2°C for every mole/liter of urea (Gelfi et al. 1994, Steger 1994). We typically use 6 M or 7 M urea as a denaturant for TTGE gels; however for sequences that require the final buffer temperature greater than 70°C, higher concentrations of urea and the addition of formamide may be needed. The TTGE run is started with the buffer temperature at the lowest or initial melting temperature and is linearly ramped to the highest or final melting temperature during the run.

The first example for calculating the temperature range is a 192 bp amplicon from the *p53* gene that contains a 40 bp GC clamp located at the 3' end. Figure 3 shows the 50% melting probability data generated by MacMelt software. The theoretical melting temperature range of the DNA sequence of interest in a nondenaturing gel is 70° to 80°C. To adjust the temperature for a urea gel, multiply the urea molarity by 2°C and subtract the result from both the high and low theoretical temperatures. In this example, the range becomes 56–66°C when 14°C (7 M urea x 2°C) is subtracted from the predicted temperature range. Under these temperature range conditions, good separation and resolution of the wild-type

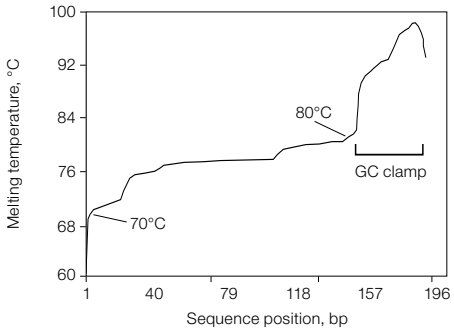

Fig. 3. The 50% melting probability for the wild-type *p53* sequence. The melting temperature range for the non-GC-clamped region is 70° to 80°C.

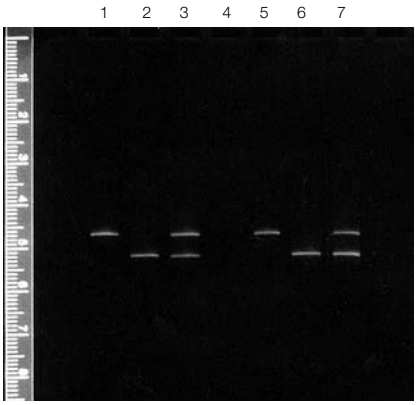

Fig. 4. Separation of mutant and wild-type *p53* samples on an 8% acrylamide/bis (37.5:1) gel containing 7 M urea. The gel was run at 130 V for 3.3 hr, 1.25x TAE buffer, temperature range of 56–66°C, and a ramp rate of 3°C/hr. Lanes 1 and 5 contains mutant *p53* (G→A), lanes 2 and 6 wild-type *p53*, and lanes 3 and 7 mutant plus wild-type *p53*.

and homozygous mutant (G→A mutation) samples are seen (Figure 4).

The second example is a 281 bp amplicon from the β-thalassemia gene that contains a 40 bp GC clamp at the 3' end. Figure 5 shows the 50% melting probability data with a narrow theoretical melting temperature range from 70° to 75°C. With a 6 M urea gel, the temperature range drops to 58–63°C after subtracting 12°C from the initial and final temperature range. Figure 6 shows the separation and resolution of the wild-type and five β-thalassemia mutant samples over this temperature range.

Temperature Ramp Rate

The rate at which temperature increases during a TTGE run is the ramp rate. The β-thalassemia and *p53* data in Figures 7 and 8 indicate that as ramp rate decreases, resolution between DNA fragments increases. This is due to the DNA fragment being exposed to the optimum denaturing conditions for a longer time period. In these examples, ramp rates of 1° to 2°C/hr gave the best resolution, but to reduce run times for routine screening, the ramp rates can be increased empirically.

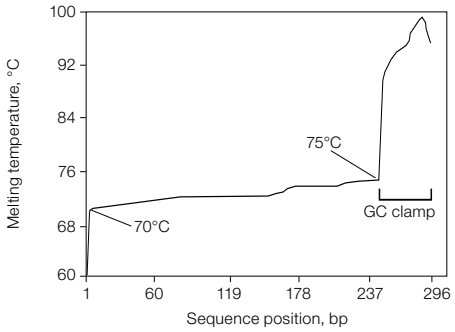

Fig. 5. The 50% melting probability profile for the wild-type β-thalassemia sequence. The melting temperature range for the non-GC-clamped region is 70° to 75°C.

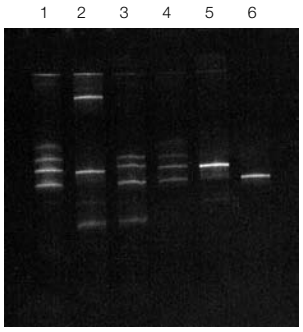

Fig. 6. Separation of wild-type and five β-thalassemia mutant samples on a 6% acrylamide/bis (37.5:1) gel containing 6 M urea. The gel was run at 130 V, for 2.5 hr, 1.25x TAE buffer, temperature range of 58–63°C, and a ramp rate of 2°C/hr. Lane 1, heterozygote IVS1-1 (G→A); lane 2, a compound heterozygote IVS1-1/IVS1-6; lane 3, heterozygote IVS1-6 (T→C); lane 4, heterozygote IVS1-110 (G→A); lane 5, homozygote IVS1-110 (G→A); lane 6, wild-type DNA.

When using a desired ramp rate for a TTGE run, the run time must be calculated by subtracting the final temperature from the initial temperature and dividing by the desired ramp rate. For example, if the temperature range is 63–66°C and a ramp rate of 2°C/hr is desired, then the run time is 1.5 hr for this gel ([66 – 63] ÷ 2 = 1.5).

Buffer Concentration

Increasing the TAE buffer concentration from the typical 1x concentration resulted in improved resolution of the DNA samples. The effect of increasing the TAE buffer concentration on β-thalassemia mutant and wild-type samples is shown in Figure 9. The gel and electrophoresis conditions were identical, except the buffer concentration varied from 1x to 1.75x. The migration rate of the DNA molecules increased with increasing TAE buffer concentrations. TAE buffer concentrations between 1.25x and 1.5x generated the largest increase in band resolution. A TAE buffer concentration of 1.25x or 1.5x improved the resolution of the homoduplex bands and heteroduplex bands, thus making it easier to identify mutants from wild-type samples.

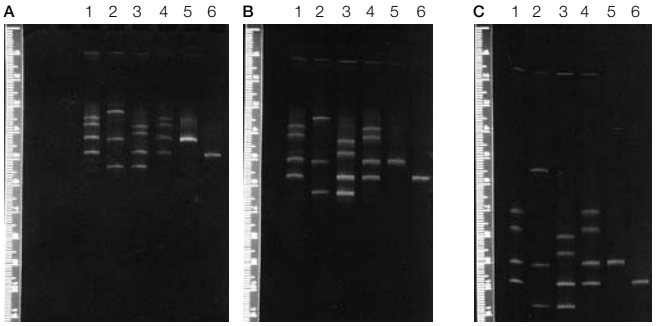

Fig. 7. Separation of wild-type and five β-thalassemia mutant samples on 6% acrylamide/bis (37.5:1) gels containing 6 M urea. The gels were run at 130 V in 1.25x TAE buffer, temperature range of 56–65°C. **A**, ramp rate of 4°C/hr and a run time of 2.5 hr; **B**, ramp rate of 3°C/hr and a run time of 3 hr; **C**, ramp rate of 2°C/hr and a run time of 4.5 hr. For all gels, lane 1, heterozygote IVS1-1 (G→A); lane 2, compound heterozygote IVS1-1/IVS1-6; lane 3, heterozygote IVS1-6 (T→C); lane 4, heterozygote IVS1-110 (G→A); lane 5, homozygote IVS1-110 (G→A); lane 6, wild-type DNA.

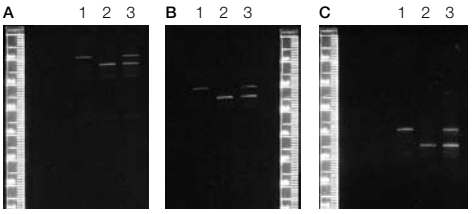

Fig. 8. Separation of wild-type and mutant *p53* samples on 8% acrylamide/bis (37.5:1) gels containing 7 M urea. The gels were run at 130 V in 1.5x TAE buffer with a temperature range of 63–66°C. **A**, ramp rate of 3°C/hr and a run time of 1 hr; **B**, ramp rate of 2°C/hr and a run time of 1.5 hr; **C**, ramp rate of 1°C/hr and a run time of 3 hr. For all gels, lane 1, mutant DNA (G→A), lane 2, wild-type DNA; lane 3, both mutant and wild-type DNA.

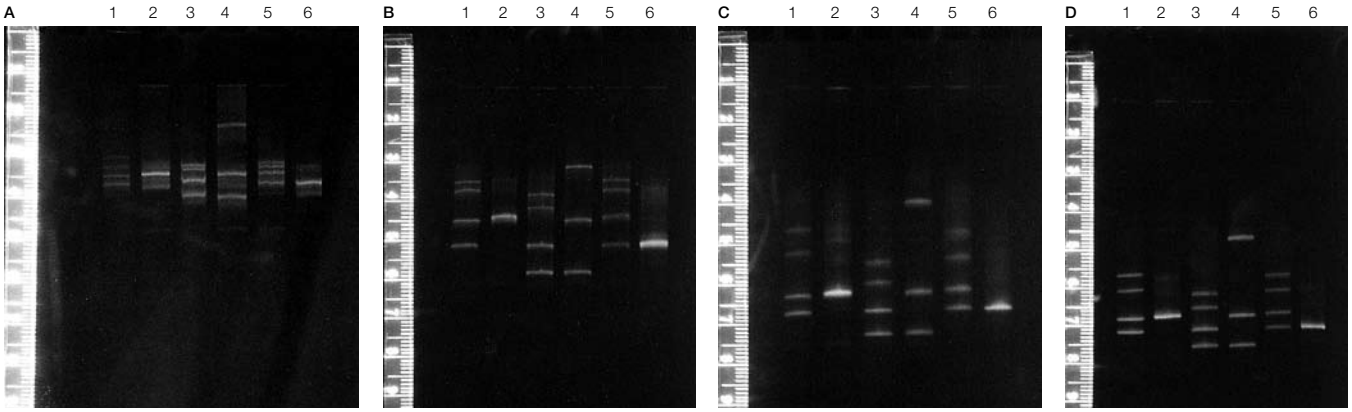

Fig. 9. Separation of wild-type and five β-thalassemia mutant samples on 6% acrylamide/bis (37.5:1) gels containing 6 M urea. The gels were run at 130 V with a temperature range of 56–65°C, a ramp rate of 3°C/hr, and a run time of 3 hr. The TAE buffer concentrations used were **A**, 1x; **B**, 1.25x; **C**, 1.5x; and **D**, 1.75x. For all gels, lane 1, heterozygote IVS1-1 (G→A); lane 2, homozygote IVS1-110 (G→A); lane 3, heterozygote IVS1-6 (T→C); lane 4, compound heterozygote IVS1-1/IVS1-6; lane 5, heterozygote IVS1-110 (G→A); lane 6, wild-type DNA.

Conclusions

The DCode instrument is designed for multiple electrophoretic mutation detection methods, including the use of TTGE to identify single-base changes. The advantages of this instrument and the TTGE technique are ease of use and the enormous potential for high-throughput screening. The DCode system can be used to screen 64 samples per run (two 32-well gels). Electrophoretic run times vary anywhere from 2 to 6 hr. Assuming a 4 hr run time, one can screen 128 samples in a day. Gel casting is easy; the constant denaturant gel used eliminates the need to pour chemical gradients gels. The gel temperature conditions can be derived from the theoretical melt profile of a wild-type sequence using the MacMelt or WinMelt software, therefore run conditions do not have to be determined empirically. Taken together, this makes TTGE a powerful technique for sequence variation studies.

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