Temperature-Programmed Capillary Electrophoresis for Detection of DNA Point Mutations


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INTRODUCTION

Detection and localization of single-base differences in specific regions of genomic DNA are of primary importance in the analysis of mutations associated with human diseases. Both inherited and acquired mutations are of importance, particularly when screening for malignant diseases. Nucleic acids are typically analyzed by electrophoretic transport through sieving matrices, since their charge-to-mass ratio is essentially constant above a length of 20 nucleotides. While mutations involving deletions are quite simple to analyze, since the mutant and wild-type PCR-amplified DNA fragments have different mobilities, point mutations result in PCR-amplified DNA fragments having identical lengths and almost identical radii of gyration, thus making them undetectable under standard electrophoretic conditions. In the latter case, the separation technique predominantly used has been denaturing gradient gel electrophoresis (DGGE), first described by Fisher and Lerman (8). It is based on variation of electrophoretic mobility of a double-stranded (ds) DNA molecule through linearly increasing concentrations of denaturing agents (typically formamide and urea). As the DNA fragment proceeds through the gradient gel, upon reaching a position at a particular concentration of denaturant, it will react as if being at the melting point ($T_m$) of its lowest melting domain. This causes denaturation, partial unwinding when amplified, the 5’-end of the duplex DNA is made to contain a high melting point region, consisting of a stretch of 40 GC pairs called GC-clamps. Since, prior to analysis, the wild-type and (presumptive) mutant chains are fully melted and re-annealed while mixed in solution, this results in a spectrum of four ds-DNA fragments: two homo- and two heteroduplexes, which can only be resolved by the DGGE technique, in which the non-isocratic conditions along the migration path are “tuned” to the slightly different $T_m$ values of the four types of duplexes present in the analyte. The $T_m$ (defined as the midpoint temperature at which each base pair is at 50/50 equilibrium between the helical and melted states) and, in fact, the entire melting profile of DNA molecules of known sequence, together with calculations of the expected changes in electrophoretic mobility in gels under denaturing conditions, can be predicted with accuracy by using the computational simulation program developed by Lerman and Silberstein (16). A variant of DGGE employs temperature gradients along the migration path and is known as TGE (18). In TGE the temperature gradient is established in the space axis before electrophoresis, usually with the aid of two thermostatic baths set at the gel extremities, either parallel or perpendicular to the migration axis.

Capillary zone electrophoresis (CZE) is rapidly emerging as a unique tool for separating a variety of charged and uncharged molecules, including proteins and nucleic acids (12). Its advantages include minute sample requirements (sample zones of just a few nanoliters), extremely high sensitivity (of the order of 10$^{-23}$ moles with laser-induced fluorescent detection) and online peak detection and integration. The applications in the field of nucleic acid analysis range from DNA sequencing (11), to analysis of PCR-amplified fragments (19) and to pulse fields for large DNAs (17). Yet, except for a few reports (4,15), CZE has been used, up...
to the present, in the isocratic mode, which does not allow one to modulate the analyte migration by altering the physico-chemical parameters in its environment. We report here the possibility of performing CZE in a non-isocratic mode, i.e., under temperature-programmed conditions (called TGCE: thermal gradient capillary electrophoresis). As a fundamental distinction, unlike TGGE, where the temperature gradient exists along the separation space and is controlled externally using circulating liquid and thermostats, the denaturing temperature gradient in the fused-silica capillaries is generated internally using ohmic heat produced by voltage ramps.

**METHODS**

**DNA Samples**

DNA specimens were obtained from cystic fibrosis (CF) patients. All patients were heterozygous for one of the following mutations in the CFTR gene: S1251N (G→A transition at position 3884 in exon 20) (13); M1V (A→G transversion at position 133 in exon 1) (5); 1717-1G→A (9); G542X (G→T at 1756) (14) and 1784delG in exon 11 (7); W57G (301 T→G) (3) and G85E (386 G→A) (22) in exon 3; and a polymorphism in exon 14a, T854T (C2694 T/G) (21). These samples were analyzed in parallel with normal controls.

**DGGE conditions**, including GC-clamped primer sequences, denaturing gradient, time and voltage of the electrophoretic separation, were as already described (3). For increased resolution, heteroduplexes were generated at the end of the PCR reaction by 5 min of denaturation at 94°C followed by 1 h of reannealing at 55°C.

**Capillary Zone Electrophoresis in Sieving Polymers**

CZE experiments were run in a BioFocus 2000 (Bio-Rad, Hercules, CA, USA) unit, equipped with a coated capillary, 60 cm long, 100 µm i.d. The background electrolyte (8.9 mM Tris-HCl, 8.9 mM borate, 1 mM EDTA and 10 mM NaCl) contained 6 M urea and 6% T linear poly(N-acryloyl amino ethoxy ethanol) (6) as sieving entangled polymer. Sample injection (electrokinetic) consisted of 3 s at 4 kV at...
different constant temperature plateaus, ranging from 53°C–68°C, according to the type of mutation under analysis. Five minutes after injection, a temperature gradient was applied, typically in the 1°C–1.5°C range, with a rather shallow sweep rate (usually 0.05°C/min). On-line DNA detection by intrinsic absorbance was at 254 nm.

Optimization of Thermal Gradients in a Capillary

Critical parameters for the success of the technique are the stability of the viscous sieving polymer, the capillary length, the choice of buffers with the correct conductivity and the choice of thermal gradients with the right slope. The temperature increments (ΔT) produced inside the capillary by given voltage gradients (E, in V/cm) can be calculated according to:

\[ ΔT = \lambda_o E^2 d^2/4\chi \]

where \( \lambda_o \) is the buffer-specific electric conductivity at a reference temperature \( T_0 \) (25°C), \( d \) is the capillary diameter and \( \chi \) is the thermal conductivity of the buffer solution. All thermal theories assume the buffer conductivity to be linearly dependent on temperature:

\[ \lambda = \lambda_o (1 + \alpha (T - T_0)) \]

where \( \alpha \) is the thermal coefficient of conductivity and \( T \) is the temperature inside the capillary (1). Thus, the experimental parameters needed for predicting the temperature increments linked to voltage ramps are as follows: the capillary diameter, its total length, the electric current values (µA) linked to a given applied voltage, the buffer electric conductivity (\( \lambda_o \)) and its thermal coefficient of conductivity (\( \alpha \)). Given these input parameters, a dedicated software allows precise determination of the inner temperature of the capillary (2). Graphs can then be easily constructed linking voltage ramps to temperature ramps.

The experimentally measured physico-chemical parameters of the buffer used are: \( \alpha = 0.0189 \pm 0.00035 \text{ K}^{-1} \) and \( \lambda_o = 2.077 \pm 0.0023 \text{ mS/cm} \). The temperature gradient that is then generated during the run takes into consideration the melting profile of the amplified fragments, as predicted by the dedicated software developed by Lerman and Silverstein (16).

RESULTS AND DISCUSSION

There are a number of rules we learned when developing the present technique: (i) The sample should be injected in the capillary so as to be maintained (by combined chemical and thermal means) just below the expected \( T_m \) value. (ii) The temperature ramp activated should be, in general, rather narrow (of the order of 1°C–1.5°C). (iii) The sweep slope should be very gentle (typically 0.05°C/min). As a starting condition, the constant denaturant concentration (6 M urea), the low ionic strength of the buffer (8.9 mM TBE) and the correct outside temperature platform at which the capillary is equilibrated produce a combined (chemical and thermal) denaturant plateau, which brings the analyte DNA fragments very close (and just below) their respective \( T_m \) values. As soon as the voltage ramp is generated, the programmed temperature increments, producing sudden mobility decrements of the duplexes, which start unwinding along the migration path, allow optimal resolution of the DNA analyte into the characteristic four-band pattern of homo- and heteroduplexes. As shown below, and contrary to what is routinely performed in gel-slab operations (where ΔT’s along the migration path as high as 15°C are typical), optimum separation in all

Figure 3. CZE analysis of 3 mutants in exon 11 of the CFTR gene: 1717-1G→A (Panel A); G542X (Panel C) and 1784delG (Panel D); the normal control (NC) is in panel E. All other conditions as in Figure 1, except that the starting temperature plateau was 56.5°C. Panel B: Plot of the temperature profile over the applied voltage ramp; Panel F: Control DGGE slab in a 10%–60% urea/formamide gradient (stain: ethidium bromide).
cases is achieved within a very narrow temperature range (1°–1.5°C). The most frequently used gradients of denaturants used in DGGE have been reproduced in the TGCE mode: 10%–60% (low melters); 20%–70% (intermediate-to-low melters); 30%–80% (intermediate-to-high melters) and 40%–90% (high melters). The optimized conditions thus developed in TGCE have been tested against the standard DGGE conditions and applied to the detection of a number of known mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, ranging from low, to intermediate, to high melters.

Intermediate-to-High and High Melters

Figure 1 shows the optimization of parameters for intermediate-to-high and high melters. Panel A shows a sample, amplified from a cystic fibrosis patient heterozygous for the S1251N mutation, injected at a constant temperature plateau (57°C), constant denaturing buffer, but in the absence of a temperature gradient. A single, asymmetric peak is obtained, surely indicative of sample heterogeneity, but hardly diagnostic. Panels B and C show the same sample analyzed in a 57°–60°C temperature interval, with a sweep rate of 0.25°C/min (B) and 0.125°C/min (C), over a wider voltage ramp (3–7 kV): almost complete loss of resolution is observed in the homoduplex region, while good resolution of the heteroduplexes is obtained (Panel B, but not in Panel C). When utilizing the correct voltage ramp (3–4 kV, with a sweep slope of 0.05°C/min), in the temperature range 57°–58°C, the expected four-peak pattern (from left to right: M/M, Wt/Wt, Wt/M, Wt/M) is obtained (Panel D), but without base-line resolution in the Wt/Wt and M/M (for wild type and mutant) doublet. Under the same set of conditions, but in the correct temperature interval (58°–59°C), the standard pattern is developed (Panel E).

Figure 2 shows the optimized condition setup for a higher melting point fragment, amplified from a CF patient heterozygous for the M1V mutation. The panel shows the electropherogram of the sample injected at a constant temperature plateau (65°C) in a constant denaturant buffer, but in the absence of a temperature gradient. We observe separation between homo- and heteroduplexes, but not within each other. The group of peaks eluting from 35 to 48 min corresponds to unpurified primers with and without GC clamps. The insert shows the optimized separation in a 65°–67°C gradient with a slope of 0.1°C/min.

Intermediate-to-Low and Low Melters

Figure 3 shows the analysis of a set of intermediate-to-low melting point fragments amplified from CF patients heterozygous for different mutations in exon 11 of the CFTR gene: 1717-1G→A (Panel A); G542X (Panel C) and 1784delG (Panel D) with their respective normal control (Panel E) and gel-slab electrophoretic profile (Panel F). All mutants exhibit the characteristic four-peak profile, vs. a single band in the control. As shown in the temperature profile of Panel B, these mutants are intermediate-to-low melters, with Tm in the 56.5–57.8°C range.

Figure 4. CZE analysis of two mutants in exon 3 of the CFTR gene: W57G (Panel A) and G85E (Panel C). Panel D: A polymorphism in exon 14a (T854 T). Panel E: Electropherogram of a normal control. Panel F gives the slab-gel patterns, obtained in a 10%–60% urea/formamide gradient in a 6.5% T, 4% C polyacrylamide slab run overnight at 16 V/cm and stained with ethidium bromide. Panel B: Plot of the temperature profile over the applied voltage ramp.
Separation of low melters is presented in Figure 4, which shows the electropherograms of two mutations in exon 3 of the CFTR gene (W57G [Panel A] and G85E [Panel C]) and of a polymorphism in exon 14a (T854T [panel D]). The analysis is performed by injecting the samples in a temperature plateau (53.5°C). All mutants are resolved in the characteristic four-peak pattern, although in the W57G mutant only apex resolution is seen. The normal control (Panel E) gives a single peak, with a transit time of 65 min (the earlier eluting peaks, from 40 to 55 min, representing primers and GC-clamps). Panel F gives the separation in a gel slab through a 10%–60% urea/formamide gradient. Panel B shows the temperature gradient profile: it spans a ΔT of only 1°C (from 53.5°–54.5°C) over a period of 20 min.

CONCLUSIONS

In conclusion, the present method, allowing for a quick and precise estimation of the existence of a point mutation in an amplified DNA fragment, might become of wide applicability in all fields of life sciences dealing with DNA analysis. In addition to the other advantages already discussed, it also offers markedly reduced analysis times: typically of the order of 70–80 min vs. 5–10 h in DGGE in slabs. Moreover, we have proven that it can cover an ample dynamic range, extending from 45°C (low melters) up to 70°C for high melters; it is thus universally applicable to any type of mutation. In cases in which some patients would carry still unidentified mutations on both chromosomes, their samples can be mixed in a 1:1 ratio with a wild-type amplified DNA in order to generate heteroduplexes. Even more easily, in case of autosomal recessive diseases, such as cystic fibrosis, samples amplified from the patient’s parents can be utilized since they both are obligate carriers for the disease. The fact that at present only single channel analysis is available should not be a deterrent, since systems with up to 100 capillaries have been already described (20) and will soon be commercially available. Moreover, CZE on a computer chip is also a reality (10). Finally, the development of other CZE systems using non-isocratic conditions will offer to the scientific community at large unexpected separation power.

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