Two-Point Fluorescence Detection and Automated Fraction Collection Applied to Constant Denaturant Capillary Electrophoresis

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ABSTRACT

Constant denaturant capillary electrophoresis (CDCE) has been shown to be a sensitive method to detect point mutations in DNA sequences of 100-bp lengths. Here, we report a significant modification for the instrumental setup that allows a highly accurate prediction of the elution time of DNA fragments from the capillary and an efficient collection of separated fractions. Fluorescently labeled DNA fragments of TP53 exon 8 wild-type and two mutants (base pair number 14480 and 14525) are detected at two separate points of the same capillary. This permits the precise calculation of the fragment velocity after separation in the heated zone because, at room temperature, all DNA fragments of the same length have the same velocity. Such precision permits the selective collection of separated fragments using an automated fraction collector for additional CDCE analysis or sequencing. Also, the two-point detection allows one to rapidly distinguish between double-stranded and single-stranded DNA fragments of the same length, a process that cannot be achieved with a one-point detection system alone. Both modifications greatly improve the procedure to detect novel mutations by means of CDCE.

INTRODUCTION

The detection of genetic changes is of central importance in molecular genetics, cancer research and human population studies. The alteration of even a single base pair in DNA can lead to changes in cellular behavior, which eventually may lead to tumorigenesis if a change occurs in crucial genes such as like oncogenes or tumor suppressor genes. Therefore, detection of such mutations would be beneficial for an early diagnosis and prognosis, and for the evaluation of therapeutic efficiency in the treatment of cancer. The possibility of detecting mutations, even of low frequencies at the level of 10^{-5} or 10^{-6} , is of particular interest for the early detection of malignant diseases, evaluation of surgical margins and monitoring relapse after cancer surgery is performed.

Constant denaturant capillary electrophoresis (CDCE) has been used to analyze low-frequency mutations in a 100-bp human mitochondrial target sequence and in sequences from antigen presenting APC and HPRT genes with naturally occurring contiguous high and low melting domains (12,15,16). Using this method, mutations have also been detected with this method in the TP53, K-ras and N-ras genes, and in additional exons of HPRT using primers with an attached GC-rich clamp to provide the necessary high melting domain (2,5,14).

CDCE is based on the principle that in DNA fragments of approximately 100-bp length and with contiguous high and low melting domains, the lower

melting domain exhibits a cooperative melting equilibrium when subjected to elevated temperature. While the high melting domain (that acts like a clamp) holds the two DNA strands together, the strands in the low melting domain denature at a temperature corresponding to its melting temperature. Therefore, the whole fragment will take up a new conformation with greatly altered electrophoretic mobility compared to the native structure. For the denaturation of the low melting domains, heteroduplexes require less thermal energy than either wild-type or mutant homoduplexes because of the base-pair mismatch that is present in heteroduplexes.

This concept was first demonstrated in the seminal work of Fisher and Lerman, who used a chemical denaturing gradient in a slab gel (6,20). An improvement of this technique eliminated the need for a gradient and replaced it with a constant denaturing condition (9). The extension to capillary gel electrophoresis subsequently increased speed and resolution of the technology, and the use of laser-induced fluorescence has increased the detection level even further (12).

This method is generally applicable to all DNA sequences containing isomelting domains of 80–120 bp when contiguous to a high melting domain. This high-melting domain may be present naturally in the sequence under study or be introduced using primers with attached GC-clamps during PCR or alternative methods. As the human genome is replete of isomelting domains—each of which represents a po-

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tential candidate sequence for CDCE—this approach becomes the method of choice for detecting both rare mutations involved in the etiology of diseases and for the detection of sequence variants in human populations like single-nucleotide polymorphisms (SNPs).

While the detection level of the instrument in its present form can be as low as a few thousand fluorescently labeled DNA molecules, the procedure is laborious, especially if the separated fractions have to be collected manually for further analysis such as amplification or sequencing. These steps are recurrent, particularly in the process of identifying novel or rare mutations. Technical improvements are needed to increase the efficiency of CDCE-based mutation detection. Here, we present methods to greatly facilitate these procedures by using a two-point fluorescence detection system and an integrated automated fraction collector.

MATERIALS AND METHODS

Instrumentation

The general layout and components used for the CDCE instrument have been described previously (5,12,14). Figure 1 shows a schematic drawing of the modified setup and the integrated fraction collector. Two-point detection was achieved by dividing the filtered laser beam (bandpass 488 ± 5 nm; Corion, Holliston, NJ, USA) from the low-power argon laser (Omnichrome, Chino, CA, USA) into two beams by a beam splitter (Type BS-550-S; Corion). Both laser beams were focused onto the capillary (focusing lenses $f = 25$ mm; Edmund Scientific, Barrington, NJ, USA) perpendicular to the microscope objectives (Oriel, Stratford, CT, USA) with the distance between both beams being 5 cm.

The fluorescence signals from each

of the windows were viewed through two 60 $\times$ microscope lenses and focused on two photomultiplier tubes (Oriel), after passing through a set of two filters (a 520-nm bandpass filter and 515-nm longpass filter; Corion). Signals from each of the photomultipliers were amplified by a current preamplifier (Model 70710; Oriel) and recorded as previously described (12,14).

Capillary Electrophoretic Conditions

A fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with 75 μ m inner diameter was coated to prevent electro-osmotic flow. The method of coating was based on Hjertén (8) and modified as described in Ekström et al. (5). The total length of the capillaries was 35 cm. Detection windows, 5 and 10 cm from the end of the capillary, were made by removal of the polyimide coating with a scalpel. The width of the detection windows was about 5 mm.

Sieving Matrix

Replaceable linear polyacrylamide (LPA) powder was obtained from Scientific Polymers from (Ontario, NY, USA) with a molecular weight of 6×10^6 . The granulated powder was dissolved very slowly in concentrations ranging of from 5%–9% in $1\times$ TBE buffer (89 mM Tris, pH 8.0, 89 mM Borate, and 2.5 mM EDTA) during gentle stirring. The LPA solutions were transferred to a 60-mL syringe (Becton Dickinson, Franklin Lakes, NJ, USA) and placed for one to two days in an upright position to allow air bubbles to rise before use. LPA was displaced from the storage syringes into a small, gas-tight 100- μ L glass syringe (VICI, Baton Rouge, LA, USA). The sieving matrix was replaced before every run to ensure the highest reproducibility and resolution.

PCR products were diluted 100-fold with distilled water and electroinjected by applying 2 μ A for 60 seconds to a 0.5 μ L sample volume of 0.5 μ L applied using a loading sleeve placed on the capillary. The sleeve was the thin part of a slim polyethylene micropipet tip that fits neatly on the capillary. The number of molecules loaded by this method was

Figure 1. Schematic sketch drawing of the instrumental setup of the two-point detection CDCE.

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calculated to be 5×10^8 copies; this calculation is based on the known number of copies produced during PCR with totally depleted primers (the number of molecules corresponds to the primer concentration used) and the sample volume loaded on the capillary.

Samples were then run at a constant voltage of 125 V/cm; the total length of the capillary was 35 cm, 10 cm of which were heated to achieve an effective separation zone for this length. The solid-state, electrically heated zone was made according to Kleparnik et al. (13). Briefly, a resistance temperature detector (OMEGA Engineering, Stamford, CT, USA) and kapton heatfilm (KH-104; Omega) were connected to an autotuneable temperature process controller (CN 76000; OMEGA Engineering) and assembled to a heat-conducting tube with a close-fit tunnel for the capillary. This setup allowed temperature control within $\pm 0.1^\circ\text{C}$ of the selected temperatures.

A fraction collector was integrated into the CDCE instrument, making it feasible to collect up to 60 fractions automatically (18). A sheath liquid flow at the exit of the capillary allowed continuous collection with an uninterrupted electrical field. The flow rate of the sheath liquid ranged between 0.83 and 1.6 $\mu\text{L/s}$. Glass capillaries (10 μL) (Idaho Technology, Salt Lake City, UT, USA) were used as collection vials and placed on a cylinder. The glass capillaries were aligned to the end of the column. A computer-controlled stepping motor moved the cylinder in small steps so that the glass capillaries were positioned towards the column at fixed intervals to collect the eluted samples for a preset length of time. Elution times were based on the assumption that the electrophoretic mobility of the DNA molecules in the column is constant at room temperature, that is, the time from the first detector to the second detector corresponds to the time from the second detector until to the end of the capillary (each distance is 5 cm).

DNA Samples

For the reconstruction experiments, PCR products of wild-type and mutant TP53 exon 8 sequences were used. In this study, the two mutants were G→A

changes at base pair 14480 and 14525, respectively (GenBank® accession no. X54156). Amplification of the wild-type and mutants was performed separately and the PCR products were mixed in precise amounts so that the mutant fractions in the samples were known.

Wild-type samples were spiked with mutant sequence at a ratio of 1/10 000 in the reconstruction experiment; heteroduplexes were converted by mass action.

PCR

PCR on both wild-type and mutant template sequences was performed using the native *Pyrococcus furiosus* DNA polymerase (*Pfu*), Stratagene, La Jolla, CA, USA) and the Stratagene-provided native *Pfu* buffer (both from Stratagene, La Jolla, CA, USA): 10 mM KCl; 10 mM $(\text{NH}_4)_2\text{SO}_4$; 20 mM Tris-HCl, pH 8.75; 2 mM MgSO_4 ; 0.1% Triton® X-100, 100 $\mu\text{g/mL}$ BSA). The 20-mer and 60-mer oligonucleotides were obtained from MedProbe, Oslo, Norway: 5'-ATCCTGAGTAGTGGTAATCT-3', 5'-Fluorescein CGCCGCGCGCGCCCGCGCCCGTCCCCGCCGCCCGCCCGCCCGTACCTCGCTTAGTGCTCCCT-3', as previously described (3,4). PCR amplification was performed with an air thermocycler (Idaho Technology), using the following program: 60 s at 94°C , 35 cycles of 7 s at 94°C , 15 s at 53°C and 15 s at 72°C . Heteroduplex formation between wild-type and mutant was promoted by heating the PCR product to 94°C for 1 min and a reannealing step at 65°C for 15 min (22). All samples were processed in duplicate.

Data Analysis and Calculations

All recorded data were processed with AcqKnowledge™ 3.0 (Biopac Systems, Santa Barbara, CA, USA). The software was used to calculate the area under the peaks to quantitate mutant fraction and to measure the migration times from the first to the second detector. The mutant fraction was calculated by summing the area of the mutant homoduplex peak with half the area of the two heteroduplexes, divided by the sum of the area from the wild-type and half the area of the two heteroduplexes. Enrichment is defined as the ratio between the mutant fraction

before and after collection.

$$\text{Mf} = \frac{\left(A_{\text{mut}} + \frac{(A_{\text{hd1}} + A_{\text{hd2}})}{2} \right)}{\left(A_{\text{wt}} + \frac{(A_{\text{hd1}} + A_{\text{hd2}})}{2} \right)}$$

Mf: mutant fraction; A: area under the peak; mut: mutant; wt: wild-type; hd1 and h2: first and second heteroduplex.

RESULTS

We have upgraded and modified a single-capillary CDCE instrument for two-point fluorescence detection and automated fraction collection. The electrophoretic separation of wild-type and mutant homo- and heteroduplexes depends on the appropriately chosen melting temperatures and the quality of the sieving matrix. For the electrophoretic runs and collections performed here, we used commercially available LPA as a sieving matrix. Different LPA concentrations were tested before the runs shown below; for the DNA samples used, we generally found separations as good as the sieving matrix conventionally used in our laboratory (21).

The separating temperature for the DNA samples used in these experiments was based on earlier findings (5) but was fine-tuned to the new temperature controller used in this experimental setup. The final temperature used for the separation was 78°C .

PCR products were diluted with deionized water before electrical injection. Aliquots of 0.5 μL diluted sample were pipetted into a loading-sleeve that allowed easy and reproducible electroinjections from small sample volumes. To completely transfer all DNA molecules in the loading sleeve to the capillary, the necessary time was determined in a series of runs. The loading voltage was applied for 5, 10, 30, 45, 60 and 120 s, respectively. The area under the curve of the product peak increased with loading time up to 45 s, corresponding to more molecules loaded into the capillary. Injection times of 60 or 120 s did not change the area under the curve, indicating that virtually no DNA molecules were left in the sleeve.

Therefore, the samples in succeeding experiments were loaded for 60 s.

Figure 2 shows a single electrophoretic run of a mixture of wild-type and mutant in homo- and heteroduplex form. The two panels correspond to the signal detected at the first and the second window. The different DNA molecules have clearly been separated in the heated zone located before the first detector. The profiles from the two detectors in this figure have been aligned for the wild-type peak to make the appearing time shift of a non-target peak (peak 5) easily visible. This peak comes from ssDNA molecules that were created during the generation of the heteroduplex molecules, as described in Materials and Methods. Because ssDNA and dsDNA have different velocities at room temperature, this time shift can immediately be seen using a two-point detection mode but

would not become apparent with only a single detection window.

The nature of this peak was known from earlier experiments in which two labeled primers were used. This ability to differentiate between ssDNA and dsDNA would be particularly helpful when heteroduplex peaks from several mutants are present, in which case the peak identification is more complicated. ssDNA is also frequently generated during PCR or in samples that are run in CDCE at high denaturation temperatures. In the original unaligned profiles, the time difference (ΔT) between the individual peaks characterizes the delay in the appearance of peaks between detection windows one and two. This delay corresponds exactly to the travel speed of the DNA at room temperature, as this portion of the capillary is unheated. While the mean ΔT for the four dsDNA peaks was 4 min 2 s, the

ssDNA peak had a ΔT of 6 min 18 s.

The instrument has previously been shown to allow detection of mutants at a ratio of 10^{-3} , or one mutant/1000 wild-type molecules (2,5,14) before any enrichment. Figure 3 shows the TP53 exon 8 wild-type spiked with the G→A mutant at base pair 14480 at a ratio of 10^{-4} (panel A). The sample was subjected to CDCE and the heteroduplex region was collected in six fractions, each of a 10-s duration (the shaded area in panel A shows the entire 60-s period). The collected fractions were re-amplified by PCR and analyzed by CDCE a second time. Panels B, C, D and E corresponding to the collected fractions 3–6 reveal the enriched heteroduplex peaks. In fraction 4, panel C, also the mutant homoduplex is also visible (peak 2). The enrichment was between 60- and 70-fold compared to the spiked sample, as shown in panel A.

Figure 2. Comparison of DNA species elution time between the two detectors. Equal migration time from detection window one to window two for all four DNA species (wild-type-WT, mutant-Mut (base pair 14525 G→A), heteroduplexes-HD) at room temperature. Upper panel, first detector; lower panel, second detector. The mean time difference (ΔT) for the four peaks is 4 minutes 42 s with one SD of 2 s. ΔT for peak number 5 is 6 min 18 s. Note the different x-axis for detector one versus detector two.

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DISCUSSION

CDCE with laser-induced fluorescence detection has become a powerful tool in mutational research. The feasibility and sensitivity of this technique have been demonstrated for several sequences, including the mitochondrial DNA (10,12), APC (16,19), H- and K ras (2,14), and TP53 (5). The principle of separation of mutants from wild-type with CDCE is based on the equilibrium melting temperature of a DNA sequence, which, in turn, depends on free energy for the denaturation of any particular base pair (6,20). Under separating conditions, a temperature inside the capillary will unwind at the low melting domain of the target sequence. Depending on the nature of the mutation, the mutant homoduplex will migrate faster or slower than the wild-type. The heteroduplexes will always migrate slower than wild-type and mutant homoduplexes because of a mismatch base-pairing in the low melting

domain. The linear detection range for laser-induced fluorescence using a single detector is from 3×10^4 to 10^{11} copies molecules (12).

When applying the two-point detection, the laser light is divided into two beams. Thus, the excitation power is reduced to 50%, compared to a single-detector system. However, this did not have any noticeable effect on the number of detectable fluorescein molecules (data not shown). This is probably because of an excess of excitation power from the laser beam on a single-detector system; consequently, splitting the laser beam will provide enough power to excite all fluorescence molecules at the two detection points.

The main advantage of two-point detection is the prediction of the elution time of peaks and the use of the calculated elution time to collect fractions of interest. This can be done by assuming that DNA travels with the same velocity from the first to the second detection window and then from the second detection window to the end of the capillary. We used the calculated elution time for the wild-type DNA to determine the collection interval for the heteroduplex region of the mixed DNA sample. The mutant fraction of the sample used was 10^{-4} , which is based on the number of mutant copies mixed with the wild-type DNA. Because this fraction is below the detection limit of 10^{-3} , no heteroduplex peaks can be seen in the profile shown in Figure 3, panel A. Using the automated fraction collector, six consecutive fractions of 10 s each were collected and the fractions subjected to PCR, followed by CDCE analysis. Four of the collected fractions contained clearly recognizable heteroduplexes, indicating an enrichment of the mutants versus wild-type. In two of the fractions (Figure 3, panels B and C), the enrichment was 60- to 70-fold, compared to the mutant fraction in the original sample. The mutant fractions were quantitated by calculating the ratio of the areas under the mutant homoduplex and the wild-type homoduplex peaks. Enrichment is calculated according to the formula given in the experimental section.

To identify an unknown mutant, a second collection was necessary, followed by PCR and sequencing of the

PCR product. A second approach to verify the mutation is to hybridize the sample with standard mutants having a similar peak pattern in the electropherogram and reanalysis by CDCE (5,7,11). The mutant standard harboring the same mutation as in the sample will display only four peaks in the profile. Detecting mutants with even lower frequencies would theoretically be possible with additional enrichments steps. However, when analyzing mutant fractions below 10^{-4} , detected mutations cannot easily be distinguished from errors introduced by DNA polymerases utilized during PCR (1,5).

The collection of *Hae*III restriction digestion fragments of PhiX-174 plasmid DNA, with UV detection close to the end of the capillary, has been demonstrated by Muller et. al. (18). After the collection of 11 fragments, each was reamplified with a specific primer set and the products were visualized by laser-induced fluorescence capillary electrophoresis. Two-point UV detection combined with collection has recently been described for capillary zone electrophoresis of a carbohydrate ladder derivatized with 1-aminopurine-3,6,8-trisulfonate (17). The authors conclude that two-point detection accurately predicts the elution time for the peak of interest.

Another important feature of the two-point detection system is that it allows the identification of target sequences based on identical velocities while excluding peaks as target peaks that do not have the same velocities. This feature is based on the fact that wild-type and mutant species migrates with the same velocity at room temperature, while incomplete PCR products, ssDNA molecules and even contaminants do not. Figure 2 shows an example of equal migration time between the two detection windows for the target sequences sequences, while the non-target peak can be identified on the basis of a different ΔT .

Setting up a two-point detection system does not require much additional instrumentation or time, compared to a single-detection CDCE system. The only custom-made part was the capillary holder. This was shaped to allow the capillary to be approached simultaneously by two microscope objectives

Figure 3. Comparison of relative fluorescence from spiked sample and PCR-amplified collected fractions. (A) Wild-type sample spiked with a mutant (base pair 14480 G→A) in a ratio of 10^{-4} . (A, gray area) The sample was subjected to CDCE and the heteroduplex region was collected in six 10-s fractions. (B–E) Fractions 3–6 reveal enriched heteroduplex peaks. Peaks numbered in fraction 4 are wild-type (1), mutant (2) and heteroduplexes (3 and 4), respectively. All profiles are adjusted to the same scale. The enrichment based on the area under the curve was between 60- and 70-fold compared to the spiked sample.

in close proximity. The cost of upgrading from a single-detection system is about 5% of the total cost of a single-point detection system.

The instrument is operated in the same manner as a single-detector instrument. Compared to a single-detector system, this system gives additional information about the nature of the peaks observed in the profiles of fractions. Peaks that do not travel with the same velocity as the wild-type at room temperature would not be considered target molecules. They can be considered contaminants, much like ssDNA or incomplete PCR products. Here, we have demonstrated a detection range of 10^{-4} mutant/wild-type, using two-point fluorescence detection in CDCE, coupled with automated fraction collection. This technique can be applied to virtually any known DNA sequence with an isomelting domain of approximately 100 bp, adjacent or attached to a high melting region. The size of the PCR product should be at least 200 bp and contain a high melting domain.

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