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Fast DNA separations using poly(ethylene oxide) in non-denaturing medium with temperature programming

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Abstract

We demonstrated fast DNA separations in low viscosity entangled solutions with a temperature gradient in a non-denaturing separation medium. The separations were carried out in a solution of commercially available poly(ethylene oxide) (PEO) [1×Tris(hydroxymethyl)aminomethane borate buffer, without urea] with a temperature gradient of 2°C/min. The performance was compared with that of a solution of PEO with urea at ambient temperature. We found that the former condition gives sufficient resolution for accurate base calling and that in general, it gave better separation for fragments larger than 450 base pairs (bp). Most importantly, the separation speed approaches 30 bp/min. In addition, we describe a simple yet reliable gel preparation protocol for such separations. © 1998 Elsevier Science B.V.

Keywords: Gel preparation; DNA; Poly(ethylene oxide)

1. Introduction

Traditionally, DNA separations have been performed using slab gel electrophoresis. Since the introduction of capillary gel electrophoresis, the field has undergone a rapid revolution [1–7]. Capillary gel electrophoresis (CGE) offers the advantage of high separation speed. This is attributed to the large surface-to-volume ratio of the capillary, resulting in effective heat removal, thus, a higher electric field (typically 300 V/cm) can be applied. Also, the possibility of scaling up to 1000 capillaries [8] makes it an attractive alternative to slab gel electrophoresis.

Theoretically, all single-stranded DNA (ss-DNA) fragments have the same charge-to-mass ratio, thus, it is not possible to separate ss-DNA fragments in a free solution. In order to achieve size-dependent separations of ss-DNA fragments, a sieving matrix must be used. Therefore, the development of an ideal sieving matrix has been the focus of the field of DNA separations. The ideal sieving matrix must fulfil the following criteria: (1) High separation speed. This is especially important to the development of high-throughput sequencing instruments to support the Human Genome Project. (2) Long read length. It will simplify sequencing an unknown piece of DNA by reducing the computational effort to assemble a finished sequence from a randomly cloned template. (3) Replaceable operation. The sieving matrix must have a low enough viscosity to efficiently fill and be pushed out of the capillary so that every run has a fresh matrix for optimum separations. This is especially important in the development of multiplexed DNA sequencers.

Early work on DNA separations by CGE was done on cross-linked polyacrylamide [1–7]. However, there are problems associated with this sieving matrix, such as gel instability over time [9], since gel replacement is not possible. Therefore, researchers

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have turned to entangled polymer solutions, such as linear polyacrylamide [10,11] and hydroxyalkyl cellulose [12]. These polymer solutions offer low-to-moderate viscosities, which make replacement of sieving matrices possible. We reported earlier on a novel sieving matrix of poly(ethylene oxide) (PEO) solution [13,14]. Its high separation speed, long read length and replaceable nature make it closely resemble an ideal sieving matrix.

In all of these reports, urea has always been added to the sieving matrices for separating ss-DNA. It functions in keeping the denatured DNA fragments from renaturing inside the capillary, which would, in turn, cause errors in base-calling. However, there are several problems associated with the use of urea. For example, it increases the viscosity of the gels and may reduce the lifetime of the capillaries, especially at high temperatures, due to the decomposition of urea.

From the point of view of thermodynamics, elevated temperatures (>60°C) should help keep the denatured DNA fragments from renaturing and avoid certain compressions (hairpins). Indeed, the commercial slab-gel protocol uses both high temperature and urea. Using CGE at elevated temperatures with gels without urea may give similar resolution as using CGE with urea. Also, it was reported that the operation of gels at elevated temperatures increases the separation speed [6,7].

The protocol for gel preparation is also important to the development of high-throughput, multiplexed systems. Usually, careful degassing is necessary to produce a bubble-free sieving matrix [15]. This is especially important if elevated temperatures are used [16]. In this paper, we report DNA separations in non-urea PEO gels with a temperature gradient. Also, a simple, yet reliable gel preparation protocol is reported.

2. Experimental

2.1. Laser-excited fluorescence detection

The experimental set-up is similar to that described previously [13]. A high-voltage (HV) power supply (Glassman High Voltage, Whitehorse Station, NJ, USA) was used to drive the electrophoresis. The entire electrophoresis and detection system was enclosed in a sheet-metal box with a HV interlock. An argon-ion laser with a 488-nm output from Coherent (Santa Clara, CA, USA, Model Innova 90) was used for excitation; the output power was 8 mW. A 520-nm cut-off filter was used to block scattered light. The fluorescence signal was transferred directly through a 10-kW resistor to a 24-bit A/D interface at 4 Hz (Justice Innovation, Palo Alto, CA, USA; Model DT2802) and stored in a computer (IBM, Boca Raton, FL, USA; Model PC/AT 286).

2.2. Elevated temperatures

The capillary was enclosed in a 1-cm I.D. water jacket. For high temperature experiments, water was maintained at 65°C by a thermobath (Cole Palmer, Vernon Hills, IL, USA) and was driven through the water jacket by a pump at a rate of 0.2 l/min. For temperature programming experiments, the water was heated at a rate of 2°C per min. The initial temperature was 35°C and the final temperature was 65°C.

2.3. Capillary and reagents

Capillaries (75 μm I.D. and 365 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). The capillaries were washed with methanol, filled with 2.5 m*M* HCl, and then electrolyzed at the running voltage (in this case, 12 kV) for 30 min prior to use. All chemicals for preparing buffer solutions were purchased from ICN Biochemicals (Irvine, CA, USA). Poly(ethylene oxide) was obtained from Aldrich (Milwaukee, WI, USA). Fuming hydrochloric acid was obtained from Fisher (Fairlawn, NJ, USA). PGEM/U DNA samples (cycle sequencing with AmpliTaq-FS polymerase, terminator-labeled) were obtained from Nucleic Acid Facilities (Iowa State University, IA, USA).

2.4. Gel preparation

2.4.1. Under air

The polymer powder (PEO: 0.2 g of M_r 8 000 000 and 0.14 g of M_r 600 000, unless specified) was stirred into 10 ml of 1×TBE buffer [89 m*M* Tris-(hydroxymethyl)aminomethane, 89 m*M* boric acid, 2

m*M* ethylenediaminetetraacetic acid (EDTA)]. The mixture was then stirred for 30 min. After stirring, the solution was placed in an ultrasonic bath for 30 min, and then degassed under vacuum for 1 h.

2.4.2. Under helium

A stream of helium gas was bubbled through the 1×TBE solution (10 ml) for 15 min and then the polymer powder was stirred into the solution. The mixture was stirred for 30 min. After stirring, the solution was placed in an ultrasonic bath for 30 min, and then degassed under vacuum for 1 h. The resulting gel solution was stored under helium gas.

2.4.3. Under reduced pressure

A 20-ml volume of 0.5×TBE buffer was stirred under reduced pressure (600 torr) for 20 min. The polymer powder (0.18 g of PEO with M_r=8 000 000 and 0.14 g of PEO with M_r=600 000) was then stirred into the degassed buffer solution. The resulting mixture was stirred for 4 to 5 h until the final volume of the solution reached 10 ml.

2.4.4. Prolonged stirring

The polymer powder was stirred into the 1×TBE buffer (10 ml). The resulting mixture was then stirred for more than 18 h. The same method was used to prepare urea gel (1.5% of PEO with a M_r=8 000 000 and 1.4% of PEO with a M_r= 600 000, 1×TBE, 3.5 *M* urea).

2.5. Capillary wall treatment

The bare capillary, typically having a total length of 45 cm (35 cm effective length) was filled with 2.5 m*M* HCl, electrolyzed at 12 kV for 30 min, and then filled with the sieving matrix (300 p.s.i., 10 min). The capillary was then equilibrated at 6 kV for 5 min prior to sample injection. The DNA sample was denatured by heating it in a denaturing solution [5:1 (v/v) formamide–50 m*M* aqueous EDTA solution] at 95°C for 3 min. The DNA was injected at 6 kV for 30 s. Capillary electrophoresis (CE) was performed at 12 kV. Between runs, the used polymer matrix was flushed out of the capillary with high pressure (2.8 MPa, 5 min), filled with 2.5 m*M* HCl, electrolyzed at 12 kV for 30 min, and then filled with new polymer matrix.

3. Results and discussion

3.1. Gel preparation protocol

We previously reported a novel denaturing gel matrix using commercially available PEO powder [13]. Briefly, the gels are made under air by stirring the powder in, followed by degassing in an ultrasonic bath and vacuum chamber. Thus, at the beginning, we used this method to prepare non-urea gels. However, with gels prepared by this method, we encountered problems with gel stability. Under high electric field strength (267 V/cm), the current drops dramatically at random times. Bubbles were usually found at around 3 cm from the injection end when we examined the capillary under a microscope. The current can be restored by trimming that portion away. However, it poses a serious problem in automatic sequencing instruments. The bubbles may be caused by ion depletion [17], which produces a highly localized electric field. As a result, excessive local heating occurs and, if there is any air present in the gel matrix, bubbles can be formed.

One way to overcome this problem is to reduce or remove all dissolved air. Therefore, we attempted to prepare the gel under a helium atmosphere because helium has a very low solubility. With this method, we have not encountered problems with gel stability under high electric field strength. However, there is another problem associated with this gel preparation protocol, i.e., the viscosity of the gel is much higher than that of the one prepared under air. This poses a serious problem in filling and refilling the capillary with the gel.

Based on the observation that it is easier to completely degas a dilute gel solution than a viscous gel solution, we started to prepare the gel using a dilute solution. This is done by doubling the amount of buffer. In this case, the solution is better degassed since the viscosity is lower. At the same time, evaporation of water from the gel solution upon prolonged stirring (4 to 5 h) under reduced pressure will produce the polymer solution of the desired concentration. Bubble formation is avoided by using this gel preparation protocol. However, this method also suffers from the same problem as above, namely, the viscosity of the gel is too high for easy filling.

We realized that completely eliminating any undissolved air may not be the answer. We have discovered an alternative but simple way to prepare sieving gels. Instead of stirring for 30 min, the gel was stirred for more than 18 h and no degassing step was employed. The gels prepared by this protocol provide the same resolution and separation speed as the ones prepared under air with degassing for the same polymer compositions (data not shown). The only differences are that the gels are less viscous and we do not encounter problems with gel stability under high electric field strength. In addition, when this type of gel was subsequently degassed in vacuum, no gas bubbles were formed. This may be due to the fact that air dissolves in the highly hydrophobic matrix, i.e. the PEO solution. The polymer network traps gas molecules and prevents them from combining together to form bubbles under excessive local heating. It is also likely that the trapped air helps to lower the viscosity of the gel by breaking some hydrogen bonds, compared with the highly viscous gels prepared under a helium atmosphere or reduced pressure. It is also possible that stirring breaks down the longer polymers. Further studies will be needed to elucidate this. One obvious advantage of this gel preparation protocol is that there is no degassing step involved at all. All one has to do is to stir the solution for a long period of time. This is thus far the simplest yet reproducible gel preparation protocol for DNA sequencing ever reported.

3.2. Capillary electrophoresis

Since there are many advantages in running CE separations at temperatures above ambient temperature, experiments were carried out at elevated temperatures (65°C). At first, the urea gel (1.5% PEO of M_r=8 000 000 and 1.4% PEO of M_r=600 000, 1× TBE, 3.5 *M* urea) was used. However, the capillary walls degraded rapidly. A brand new capillary can only be used twice despite efforts to clean and regenerate the inner surface between runs. Urea is known to decompose at high temperatures. However, in literature reports based on linear polyacrylamide and coated capillaries, runs at high temperature did not seem to irreversibly damage the capillary walls. When heated, urea decomposes to ammonia, as confirmed by the pungent smell in such solutions. Apparently, some specific reactions between PEO and the degradation products of urea cause PEO to adhere less well to bare fused-silica [18], thereby compromising the separation efficiency. One function of urea is to prevent the denatured DNA from renaturing and to remove compressions; running the separations at elevated temperature should keep the DNA in the denatured state. Thus, a non-urea sieving matrix was used for CGE at elevated temperatures. Because the non-urea gel has a lower viscosity than that of a urea gel of the same composition, a gel matrix with a higher proportion of PEO (M_r= 8 000 000) can be prepared. Therefore, one gets better resolution of the larger fragments while keeping a reasonable viscosity for easy filling. A solution of 2.0% PEO (M_r=8 000 000) and 1.4% PEO (M_r= 600 000) provides the optimum separation and viscosity. At room temperature, its viscosity is similar to that of the urea sieving matrix with a viscosity of 1200 cp [13]. All of the non-urea gel experiments described below were performed with this particular matrix, unless specified otherwise.

Fig. 1a shows an electropherogram of the DNA Sanger ladder [base pairs (bp) 23 to 80] at 65°C, and Fig. 1b shows an electropherogram of the same sample at 25°C. There are some regions (marked by triangles) where ambient conditions gave better resolution than achieved at elevated temperatures. These smaller fragments are moving too fast to be well resolved. The only exception is at the location marked by an asterisk. In this region, the peaks are well resolved at elevated temperatures (A/G peaks are not resolved, which will be explained later), but not at ambient temperature. This is due to the formation of hairpins in the DNA fragments under ambient conditions in a non-denaturing medium. It is well known that G/C rich regions are more susceptible to compression. This indicates that performing CGE at elevated temperatures can function in the same way as found when using urea. We are not aware of previous work on DNA sequencing without urea, although somewhat lower concentrations of urea (<8 *M*) compared to slab gels seem to be adequate. We attribute our observations to the inherent nature of PEO vs. polyacrylamide. That is, PEO itself partially serves as a denaturing agent. Our test sample, PGEM/U, is known to be particularly prone to hairpin formation due to its high GC content in

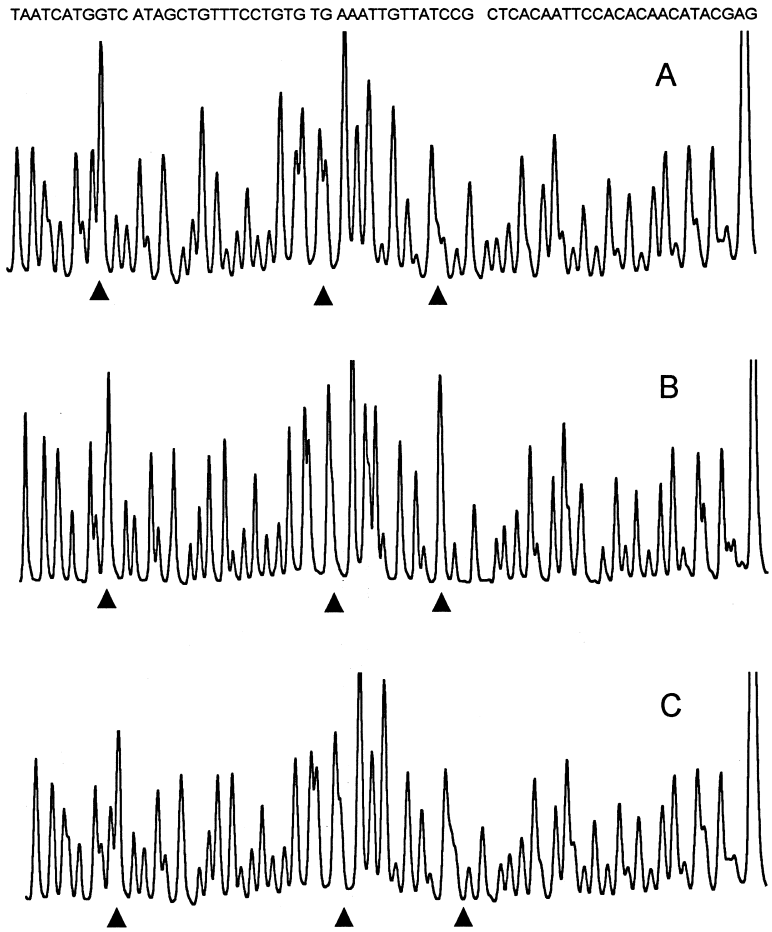

Fig. 1. Comparison of the separation of DNA Sanger fragments from base 23 to base 80 by CGE. The span of the abscissa is different in each case and is specified below. The top label shows the corresponding sequence of the fragments. (A) Non-urea PEO gel at elevated temperature (65°C), 8.1–10 min; (B) non-urea PEO gel at ambient temperature, 13.4–16.6 min; (C) non-urea PEO gel with temperature programming (35–65°C at 2°C per min), 10.3–12.6 min.

certain regions. Future studies involving even more challenging GC-rich DNA samples are planned to test this idea. Fig. 2a–b show the electropherograms of the DNA Sanger ladder (bp 116 to 182) at 65 and 25°C, respectively. It clearly shows that much better resolution is achieved at elevated temperatures than at ambient temperatures (marked by triangles).

From Figs. 1 and 2, one finds that the non-urea gel matrix provides better resolution for small base pairs at room temperature, but higher resolution for longer base pairs at elevated temperatures. A temperature gradient can then combine the advantages of both ambient and elevated temperature conditions. This was used to produce the electropherograms shown in Figs. 1c and 2c. The starting temperature was 35°C, which was gradually increased to 65°C (the final temperature). It can be seen that the resolution of smaller fragments (Fig. 1c) is similar to that obtained under ambient conditions, while the resolution of larger fragments (Fig. 2c) is similar to that obtained at elevated temperature. It is interesting to note that the region marked by asterisks has similar resolution to that region at elevated temperatures (Fig. 1c). In addition to the high resolution, the other advantage of temperature programming for the non-urea gel matrix is the high separation speed. The total running

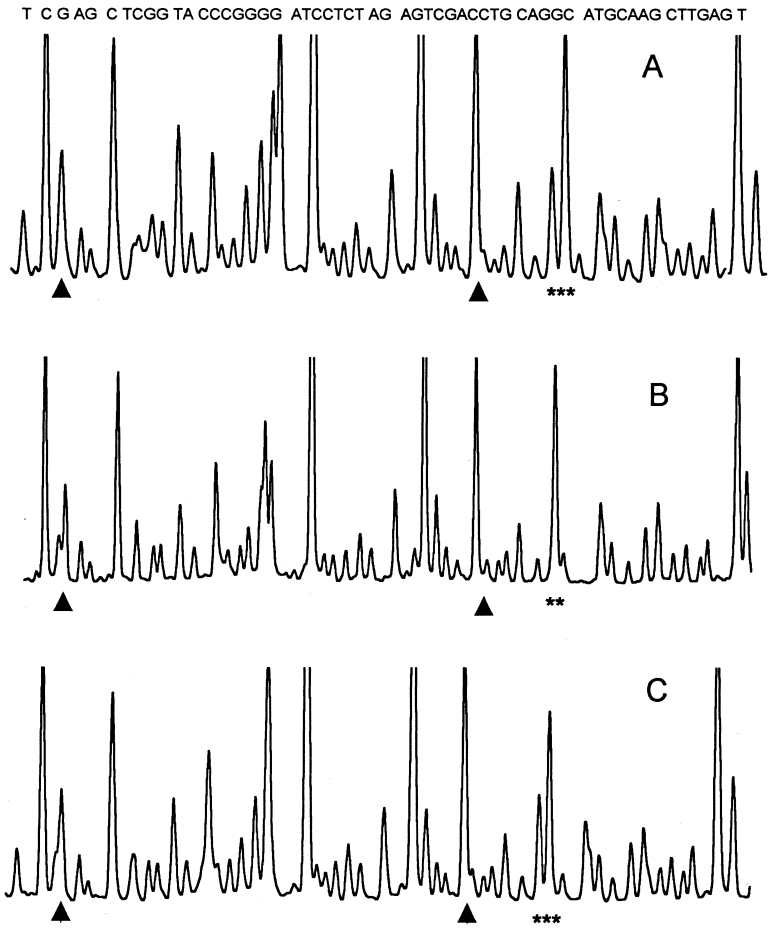

Fig. 2. Comparison of the separation of DNA Sanger fragments from base 116 to base 182 by CGE. The span of the abscissa is different in each case and is specified below. The top label shows the corresponding sequence of the fragments. (A) Non-urea PEO gel at elevated temperature (65°C), 10.9–13.1 min; (B) non-urea PEO gel at ambient temperature, 18.2–22.2 min; (C) non-urea PEO gel with temperature programming (35–65°C at 2°C per min), 13.6–16.2 min.

time from bases 30–420 is 13 min, leading to an average separation rate of 30 bp/min.

Figs. 3 and 4 are electropherograms of the DNA Sanger ladder in a non-urea gel with temperature programming and in a urea gel at ambient temperature, respectively. From Fig. 4 (>500 bp), it is clear that more fragments are resolved in the non-urea gel. From Figs. 3a and 4a (bp 57 to 105), it can be seen that we found overlap between some peaks in the non-urea gel electropherograms. This is because we used a shorter capillary for the non-urea gel experiments (45 cm total length compared to 60 cm total length for urea gel experiments). The shorter capillary resulted in faster separations. However, we confirmed that this extent of peak overlap does not pose any problems for base calling.

It is well known that dyes impose a mobility shift on the DNA fragments, due to their charge and interactions with the sieving matrix. In our case, we found that As move slower, while Gs move faster than average. Fig. 5 is an expanded view of part of the electropherogram (bp 76 to 91) shown in Fig. 3a. We can clearly see the gaps before As and after Gs (marked by asterisks). In fact, this is also the reason for some overlap of A/T and A/C peaks over the entire region. In addition, the uneven peak heights

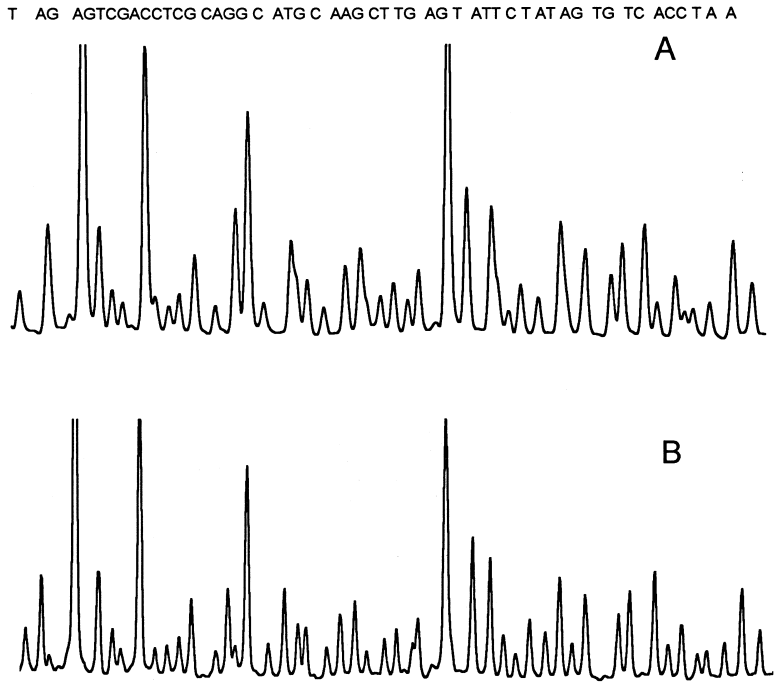

Fig. 3. Comparison of the separation of DNA Sanger fragments from base 57 to base 105 by CGE under different denaturing conditions. The span of the abscissa is different in each case and is specified below. The top label shows the corresponding sequence of the fragments. (A) Non-urea gel with temperature programming (35–65°C at 2°C per min), 11.4–13.3 min; (B) urea gel at ambient temperature, 19.3–22.7 min.

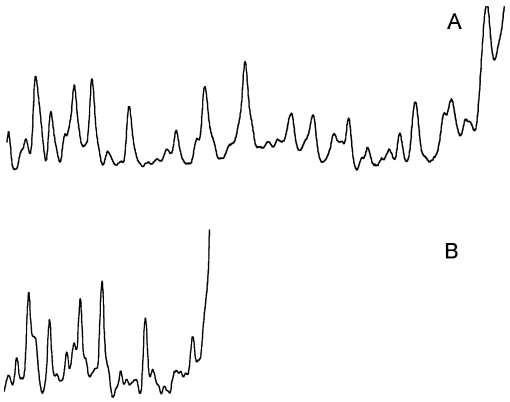

Fig. 4. Comparison of the separation of DNA Sanger fragments for >500 bases by CGE under different denaturing conditions. The span of the abscissa is different in each case and is specified below. The top label shows the corresponding sequence of the fragments. (A) Non-urea gel with temperature programming (35–65°C at 2°C per min), 29–35 min; (B) urea gel at ambient temperature, 49.1–52.0 min.

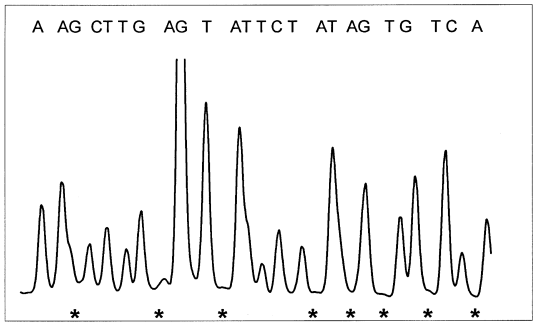

Fig. 5. Separation of DNA fragments by CGE with a non-urea gel and temperature programming. The top label shows the corresponding sequence of the fragments from base 76 to base 91 (12.2–13.1 min). The gaps before As and after Gs are marked by asterisks.

caused by this polymerase chain reaction (PCR) enzyme, AmpliTaq FS, and the use of dye-labeled terminators add another complication to the situation [19]. It is known that A is always followed by a weak G for this cycle-sequencing protocol. These two factors together account for the overlap of A/G

peaks. These peaks can be resolved by modifying the base-calling program to include the mobility shift of the As and Gs, as done in commercial software. These regions are easy to recognize, since most overlapped peaks have a larger width than that of a single peak. Preparing the DNA fragments with dye-labeled primers and an enzyme such as Sequenase can be used to overcome the problem of peak–height variations. Another way to overcome this problem is to develop a set of dyes that have similar mobilities [20,21].

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