DNA Diagnosis using BioFocus® 3000 and LIF² Laser Induced Fluorescence Detector

Michiei Oto, Department of Biotechnology, Tokyo Technical College, 1-15-5 Higashi, Kunitachi-shi, Tokyo 186, Japan

Introduction

It is now clearly understood that various diseases, including cancers, result from alterations at the genetic level. Therefore, DNA diagnosis by means of molecular biology techniques is becoming an increasingly useful tool for routine clinical analysis. PCR-based analysis of genetic alterations has been applied to the diagnosis of various diseases,[1] however, for this technique to become widely accepted as a tool for clinical DNA diagnosis, the development of an automated analytical system is essential. In the pre-PCR steps, automated DNA extraction methods and robotic manipulation have been developed to support routine applications and are commercially available. In contrast, for post-PCR steps, the manual methods of gel electrophoresis followed by ethidium bromide or silver staining are still generally performed to separate and detect nanogram to picogram quantities of DNA.[1] However, as gel electrophoresis is labor intensive and difficult to automate, it is not suitable for routine diagnosis.

In contrast to gel electrophoresis, capillary electrophoresis (CE) is a promising method for the rapid and reproducible separation of DNA and it can be used to fully automate the DNA diagnostic process.[2] This technique can separate DNA fragments ranging from 70–10,000 bp in size within 20 minutes,[3] hence it is useful for the analysis of PCR products.[2]

There are two major types of CE used for the analysis of DNA; gel sieving and non-gel sieving. Gel sieving CE offers very high resolution separations but suffers from the disadvantages of poor reproducibility and reusability, because of the difficulty of gel preparation. Non-gel sieving CE is a specialized form of capillary zone electrophoresis performed with a buffer containing polymer additives, such a methylcellulose.[4] These additives, when combined with the separation buffer at a sufficient concentration, form a sieving matrix which retards DNA species on the basis of their molecular mass. The non-gel sieving matrix can be quickly prepared prior to each sample by simply filling the capillary with buffer containing the polymer additives. The capillary is then purged and the matrix replaced between samples to insure highly reproducible results and a reusable capillary.[2, 4]

Data acquisition and analysis are computer-controlled, which facilitates automated DNA detection in multiple samples and direct UV/Vis or laser induced fluorescence detection removes the requirement for an external staining procedure.

Traditionally, extensive purification of amplified DNA by ultrafiltration or phenol extraction has been performed before CE analysis, to avoid peak broadening and interference resulting from the presence of proteins and high salt concentrations. An additional purification step is, however, a disadvantage, especially when analyzing a large number of samples. We investigated conditions permitting the direct use of unpurified PCR products in CE analysis. The BioFocus 3000 CE system (Figure 1) has been successfully used in these experiments to automate the analysis of not only double-stranded but also single-stranded DNA. The BioFocus system allows the injection of viscous buffer containing polymer-additives into the capillary using high pressure (100 psi) and can control the capillary temperature at a constant level. These features make the instrument ideal for non-gel sieving CE. Here, we describe various methods for the analysis of PCR products with a BioFocus 3000 system, and their application to DNA diagnosis.

Fig. 1. BioFocus 3000 capillary electrophoresis system.

BIO-RAD

Specimens and Genomic DNA Preparation for CE Analysis

Tumor specimens from patients were obtained by means of surgical resection. High molecular weight DNA was prepared by the method described previously.[5] Lymphocyte DNA from the respective patients was prepared similarly.

DNA Amplification Using the Modified Rapid Cycle PCR Technique

A rapid cycle PCR technique has been reported,[6, 7] which requires specialized equipment. We modified the rapid cycle PCR technique by using a regular program incubator. Oligonucleotide primers (160 nmol/L), 20–200 mmol/L dNTP, 0.5 units of Taq DNA polymerase (Wako Chemical Co., Ltd., Japan), 10 mmol/L Tris-HCl (pH 8.8), 5 0 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.1% gelatin were mixed in a thin-walled tube (Robbins Scientific Co., Ltd.), and a piece of wax (AmpliWax™, Perkin-Elmer Cetus Instruments Co., Ltd.) was added. To melt the wax, the tube was subjected to 70 °C for 1 min and subsequently kept at room temperature to re-solidify. To obtain constant results as required for routine assaying, multiple tube preparations were stored at 4 °C until required for PCR experiments. 1–5 µl of template DNA was placed on the wax surface of the previously prepared reaction tubes (Figure 2) and 40 reaction cycles of 94 °C for 10 sec, 60 °C for 20 sec and 72 °C for 10 sec were carried out in a program incubator, requiring approximately 1 hour.

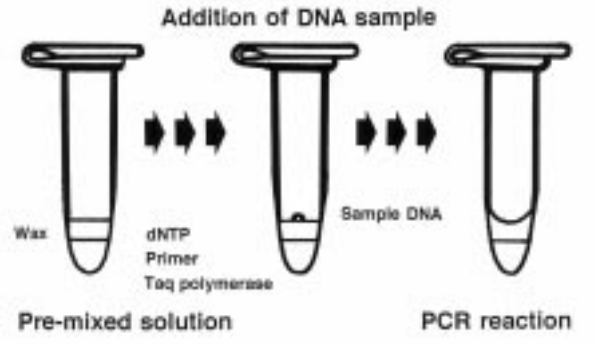

Fig. 2 Modified rapid cycle PCR technique. A DNA sample was added to a pre-mixed solution covered with wax and then subjected to the PCR reaction.

By gel electrophoretic analysis, the amplified DNA obtained with this method was determined to be of the same quality as that obtained with the regular PCR method (data not shown). DNA amplified with the pre-mixed reaction solution after storage for 1, 2 and 4 months at 4 °C produced similar results to that obtained with a fresh pre-mix preparation (data not shown). In routine experiments, after amplification the solid wax plug was pierced with a pipette tip and the amplified products were removed and subjected to CE analysis without further purification.

Optimization of the CE Conditions

CE was performed with a BioFocus 3000 system, using a 50–75 µm ID x 36 cm long linear polyacrylamide (LPA) coated capillary. Carousels with capped vial holders allowed the analysis of 30 samples consecutively. Prior to sample analysis, the running buffer was filtered and the samples were degassed by centrifugation. Pressurized injection at 5 psi for 12 seconds was performed to introduce 10 nl of each DNA sample for analysis. The electrophoresis was performed for 15–20 minutes at 139–556 V/cm at 20–40 °C in TBE buffer (0.267 M Tris-borate, pH 8.3, 1 mM EDTA) containing polymer additives (Bio-Rad Laboratories). The UV detector was set at 260 nm, with a range of 0.02 absorbance units.

In order to optimize resolution, the separation of a DNA standard (pBR322/AvaII/EcoRI, 222–1,746 bp; Bio-Rad Laboratories) was analyzed over a range of voltages and temperatures. Higher voltages yielded shorter run times but at the expense of resolution. To analyze 200–300 bp DNA products rapidly, sufficient resolution was obtained at 222 V/cm in 10 min (Figure 3). Better resolution was achieved using a field strength of 139 V/cm but with an unacceptable analysis time of close to 25 minutes. To determine the optimal temperature for the 222 V/cm separation, the standard DNA was analyzed across a series of temperatures from 20–40 °C. The best separation was obtained at 30 °C (data not shown).

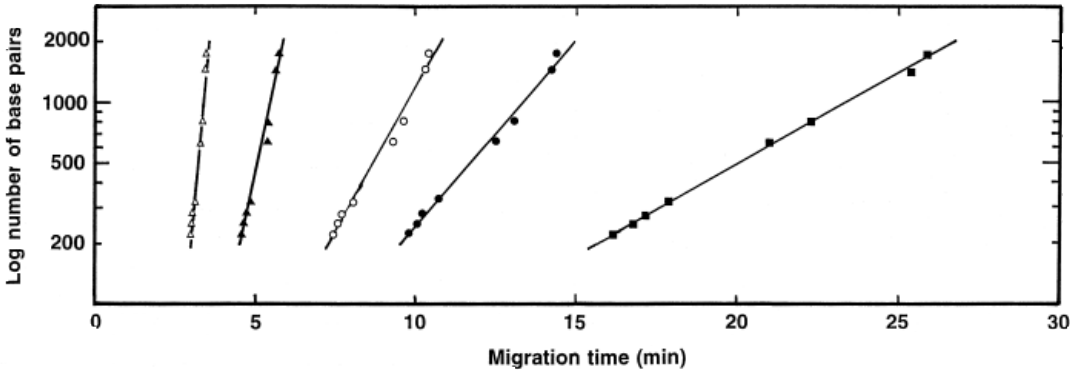

Fig. 3. Effects of voltage on the analysis time and resolution of standard DNA (pBR322/AvaII/EcoRI). The electrophoretic voltages were 139 V/cm (■), 222 V/cm (●), 278 V/cm (○), 417 V/cm (▲), and 556 V/cm (△), respectively. The running temperature was 30 °C. Other conditions are listed under Materials and Methods.

Direct Application of PCR Products to CE

Prior to CE analysis, PCR products are typically purified by ultrafiltration or phenol extraction to avoid peak broadening and interference due to high concentrations of salt in the samples. However, these steps are disadvantageous for the analysis of a large number of samples. For application to routine DNA diagnostics, we examined the use of unpurified PCR products in CE based analysis. When unpurified PCR products which were produced under ordinary conditions were analyzed, extra peaks were found in front of the target peaks (Figure 4A). To study the source of these extra peaks, the 200 µM dNTP and 160 nM primer components of the PCR reaction mixture were analyzed separately. The 200 µM dNTP produced the same pre-target peaks as those observed in the unpurified PCR sample, while the 160 nM primers did not generate any peaks (data not shown). Therefore, PCR was carried out using a reduced dNTP concentration of 20 µM. The resultant PCR products produced the same target peaks, but had no additional pre-target peaks (Figure 4C). Excessive peak broadening due to the difference in salt concentration between the PCR products and the low salt running buffer was not observed (data not shown). The data indicate that PCR products with 20 µM dNTP can be subjected to CE without further purification.

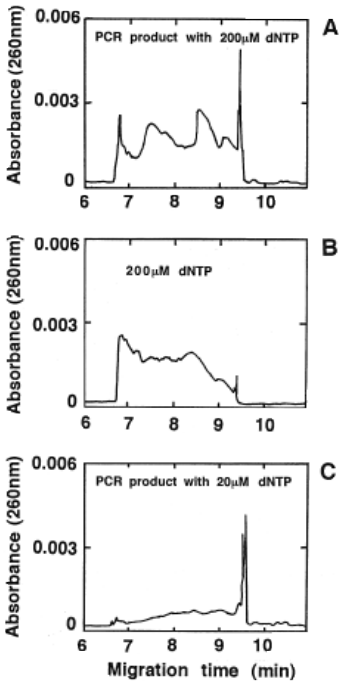

Fig. 4. Effect of the dNTP concentrations on the PCR reaction products.

In order to define the reproducibility of this technique, three independent PCR products, ranging in size from 200 to 300 bp, were amplified and each subjected to 10 separate CE analyses. The CV of the migration time was shown to be within 0.7% (data not shown), indicating good reproducibility.

Examples of DNA Diagnosis with a BioFocus 3000 System

DETECTION OF MUTATED C-H-RAS BY THE PCR-RFLP TECHNIQUE

PCR-RFLP is the most popular technique for detection of point mutations in a target gene. In this experiment the DNA fragment (63 bp) including codon 12 of the c-H-ras oncogene was amplified with specific primers (Figure 5A). PCR products were digested with MspI and then subjected to CE. In normal cells, the GGC sequence in codon 12 was digested with MspI and one peak containing 31 and 32 bp fragments appeared in the electropherogram, while in the T-24 cell line (derived from a bladder cancer patient), the GGC sequence in codon 12 had changed to GGT and thus was not digested by MspI. As a result, a single peak corresponding to a 63 bp product appeared in the electropherogram (Figure 5B).

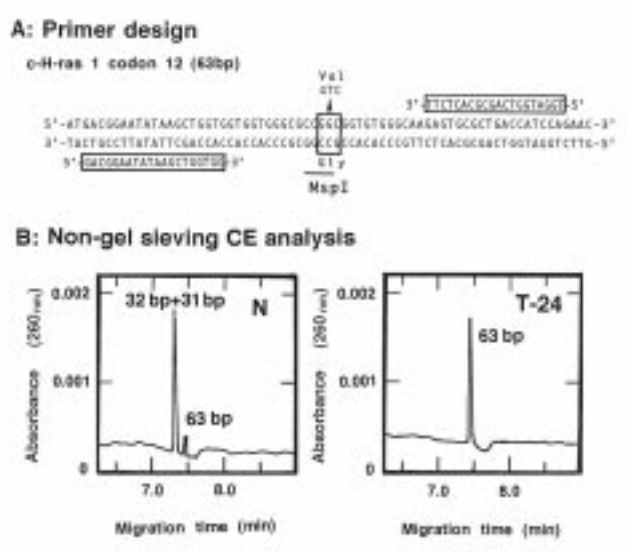

Fig. 5. Detection of the point mutation of c-H-ras Codon 12 using the PCR-RFLP technique.

DETECTION OF MUTATED C-K-RAS BY THE LCR METHOD

To detect a point mutation of codon 12 of the c-K-ras oncogene, four types of specific primers (Figure 6A) were synthesized. The DNA fragment (58 bp) containing codon 12 was amplified by the ligase chain reaction (LCR) technique[8, 9] using thermostable ligase. The fragment from normal cells containing GGT in codon 12 was amplified, while the one from A431 cells containing AGT in codon 12 was not amplified. CE analysis was successfully used to determine whether the 58 bp fragment was amplified by the LCR technique through the simple presence or absence a peak (Figure 6B).

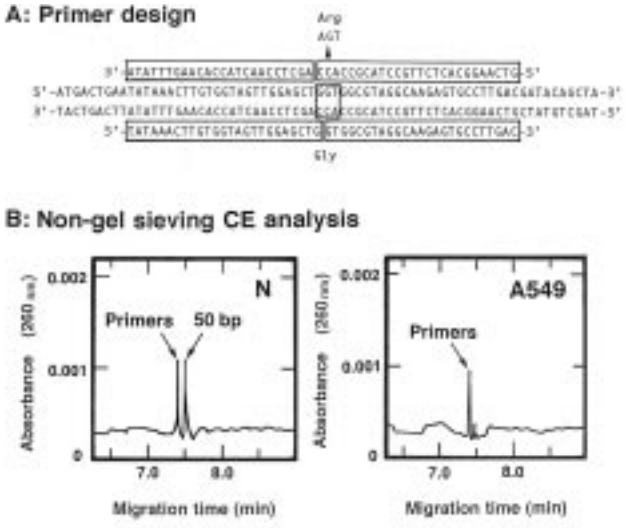

Fig. 6. Detection of the point mutation of c-K-ras codon 12 using the LCR technique.

IDENTIFICATION OF MUTATED P53 IN CANCERS BY THE PCR-SSCP TECHNIQUE

Point mutations in the tumor suppressor gene, p53, have been linked to many human cancers.[10] Therefore, for cancer diagnosis it is very important to detect alterations in the p53 gene. Single strand conformation polymorphism (SSCP) analysis is the most widely used technique for detecting such genetic changes.[11, 12] In this experiment the DNA fragment (32 bp) of p53 exons 5–6 was amplified under optimal conditions with specific primers, denatured at 94 °C for 5 minutes, chilled on ice and then subjected to non-gel sieving CE directly without further purification.[12] DNA samples were electrophoresed for 30 min at 139 V/cm at 25 °C in PCR Product Analysis Buffer (Bio-Rad). On analysis of the normal DNA derived from a colorectal cancer patient, two SSCP peaks were observed in the electropherogram. In contrast, tumor DNA acquired from the same patient produced a different electrophoretic profile with additional SSCP peaks derived from the mutant DNA (Figure 7A). To determine the DNA sequences of the peak materials, the first single strand peaks of the normal and tumor DNAs were collected and used as templates for cycle sequencing.[12] In codon 175 of the normal DNA, a CGC sequence was identified, while CAC was observed in the tumor DNA (Figure 7B).

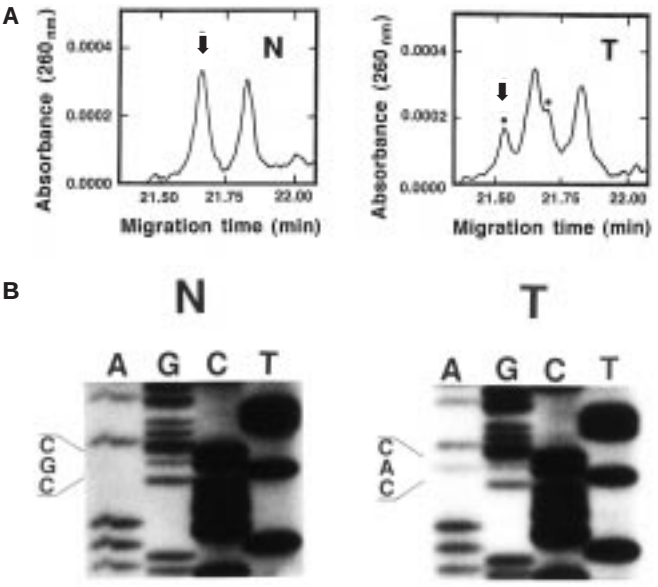

Fig. 7. SSCP analysis of the human p53 gene derived from normal DNA (N) and tumor DNA (T). (•) indicates additional peaks of tumor DNA and (⬇) indicates the peaks collected for sequencing.

DETECTION OF MICROSATELLITE INSTABILITIES IN COLORECTAL CANCERS

Recently, microsatellite instability has been shown to be relevant to various human diseases, including fragile-X syndrome.[13] Huntington's disease[14] and several human cancers.[15] In colorectal cancer, the abnormalities of D2S123, (CA)n repeats have been reported to be effective markers of microsatellite instability.[15, 16] To detect the (CA)n repeat abnormality, D2S123 DNA from colorectal cancer patients was amplified by the PCR technique and then subjected to non-gel sieving CE.[17]

The results of CE analyses of amplified D2S123 DNA derived from various colorectal cancer patients are shown in Figure 8. The electrophoretic profile of the amplified products was compared between tumor and normal DNA from the same patient. In case A, the mobility of tumor DNA was faster than that of normal DNA, and in case B the largest DNA fragment observed in normal cells had disappeared, however, in case C the mobilities of tumor and normal DNA were identical. The disappearance of the large fragment in the tumor DNA of case B may indicate a loss of heterozygosity (LOH). However, further studies are necessary to determine whether the phenomenon observed is due to genetic instability or LOH. The data indicate that the microsatellite instability observed in some cancers could be detected in approximately 10 minutes using CE techniques.

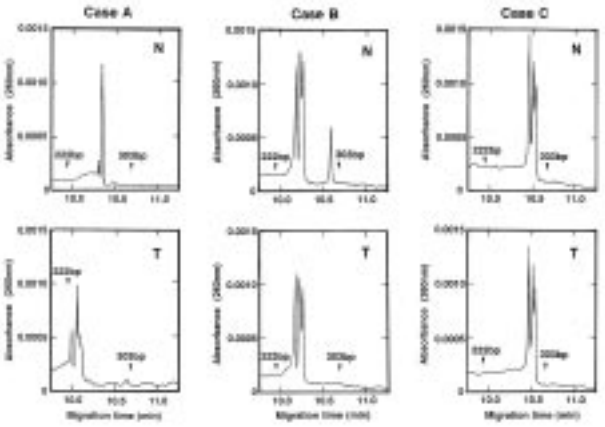

Fig. 8. Analysis of (CA)n repeats amplified by the modified rapid cycle PCR method from various specimens. Analysis by non-gel sieving CE.

ANALYSIS OF THE (A)10 REPEAT OF THE TGF-β TYPE II RECEPTOR GENE

One or two base deletions in the polyadenine tract, (A)10 repeat, of the TGF-β type II receptor (TGF-β RII) gene are detected in 90% of hereditary non-polyposis colorectal cancers and some sporadic colorectal cancers with microsatellite instability[18, 19, 20] indicating that the (A)10 repeat is a hot spot for mutation. Therefore, TGF-β RII may be one of the target genes of defective DNA repair and analysis of the (A)10 repeat should greatly facilitate DNA diagnosis of some cancers.

To analyze the abnormality of TGF-β RII, the (A)10 repeat was amplified by the modified asymmetric PCR technique.[21] On CE analysis of DNA derived from normal mucosa of a colorectal cancer patient, the (A)10 repeat peak was observed in the electropherogram, while tumor DNA obtained from the same patient showed the presence of an additional (A)9 repeat peak derived from a single nucleotide deletion the (A)10 repeat in TGF-β RII (Figure 9).

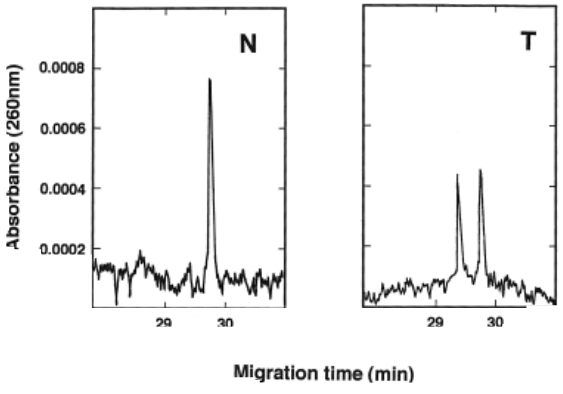

Fig. 9. Detection of the deleted (A)10 repeat of the TGF-β type RII gene in colorectal cancers. The electropherograms are for normal DNA (N) and tumor DNA (T) from the same patient.

These data demonstrate that the amplified (A)10 repetitive sequence of TGF-β RII genomic DNA can be analyzed by non-gel sieving CE within 30 minutes. This protocol can also clearly distinguish whether specimens have a homozygotic or heterozygotic allele by the number of specific peaks in the resultant electropherogram.

DNA Diagnosis with the BioFocus LIF² Detector

In cancer specimens, especially in formalin-fixed, paraffin-embedded tissues, it is often found that the amount of DNA amplified by PCR is low due to reduced DNA content of the cancer cells and DNA degradation. In such cases, target DNA should be analyzed using high sensitivity fluorescence detection. The BioFocus LIF² detector has been shown to be an effective instrument for detecting very small amounts of DNA with high sensitivity and resolution.

To evaluate the relative sensitivity and background of this detector in comparison to UV detection, an analysis of the (A)10 repeat of TGF-β RII using the BioFocus LIF² was conducted. A serial dilution of the PCR product containing the (A)10 repeat sequence was analyzed (Figure 10). In comparison to UV detection, DNA was detected with much greater sensitively and lower background using the LIF detector. The advantages of enhanced sensitivity LIF detection are clearly shown in Figure 11. Using UV detection it is difficult to determine whether clinical samples A and B from formalin-fixed, paraffin-embedded tissues had a homozygotic or heterozygotic allele of the (A)10 repeat. However, when analyzed using the LIF² detector, it is obvious that both samples A and B had heterozygotic alleles.

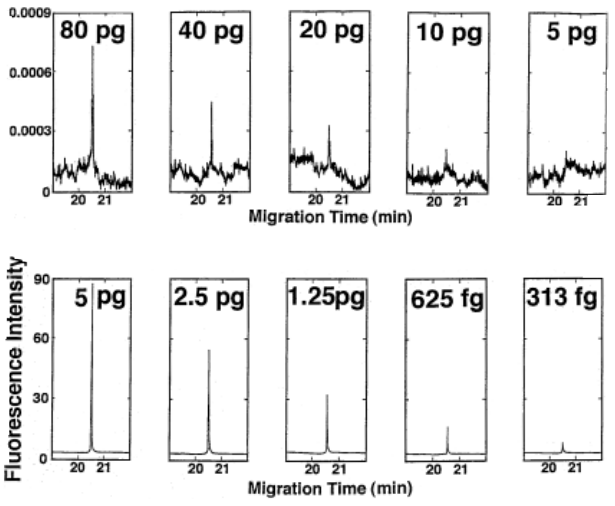

Fig. 10. Sensitivity comparison between UV and Laser Induced Fluorescence detection for a serial dilution of the (A)10 repeat of TGF-β RII. (UV top, LIF bottom).

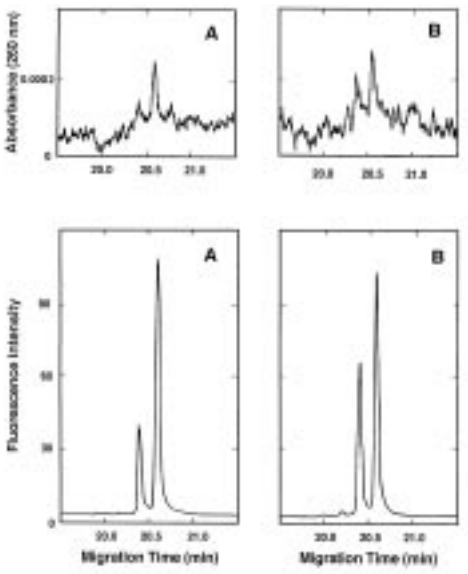

Fig. 11. Heterozygosity analysis of two formalin fixed, paraffin-embedded tissue samples. (UV top, LIF bottom).

Expansion of the application of CE to routine DNA diagnosis will require this technique to rapidly examine a greater number of DNA samples simultaneously and with high sensitively. Two channel LIF detection will meet these demands. Using the BioFocus LIF² system, not only can different DNA markers but also different tissue specimens (*e.g.* normal and cancer tissues) from the same patient be simultaneously analyzed using two unique fluorescent dyes (*e.g.* FITC and Texas Red). This method could also be applied to increase throughput when detecting point mutations using either RFLP or SSCP techniques.

The Future of CE

CE can analyze DNA with higher resolution and be performed in a shorter time than traditional gel based electrophoresis, as shown by the data above. In particular, CE is a powerful tool for the analysis of one or two base differences in microsatellite DNA.[17, 21] Moreover, the automation of CE is feasible and it can be easily applied to the routine testing of DNA. Because the repetitive sequences seen in microsatellite DNA and variable number of tandem repeat (VNTR) DNA can be analyzed with a higher degree of resolution, CE might be effectively applied to not only DNA diagnosis but also to forensic science, and the identification of plants, fish and other organisms. In the future, CE instruments will offer enhanced features and hold even greater potential as tools for the analysis of various applications in routine DNA diagnosis.

References

1 *Rossiter, B. J. F. and Caskey, C. T., Clinical application of the polymerase chain reaction, in Mullis, K. B., Ferre, F. and Gibbs, R. A. eds, The Polymerase Chain Reaction, Boston, Birkhauser, 395–405 (1994).*

2 *Martin, F., Vairelles, D. and Henrion, B., Automated ribosomal DNA fingerprinting by capillary electrophoresis of PCR products,* Anal. Biochem., *63, 182–189 (1993).*

3 *Strege, M., Laguinitin, Separation of DNA restriction fragments by capillary electrophoresis using coated fused silica capillaries,* Anal. Chem., *63, 1233–1236 (1991).*

4 *Zhu, M., Hansen, D. L., Burd, S. and Gannon, F., Factors affecting free zone electrophoresis and isoelectric focusing in capillary electrophoresis,* J. Chromatogr., *480, 311–319 (1989).*

5 *Blin, N. and Stafford, D. M., A general method for isolation of high molecular weight DNA from eukaryotes,* Nucleic Acids Res., *3, 2303–2308 (1976).*

6 *Wittwer, C. T., Fillmore, G. C. and Hillyard, D. R., Automated polymerase chain reaction in capillary tubes with hot air,* Nucleic Acids Res., *17, 4353–4357 (1989).*

7 *Wittwer, C. T., Fillmore, G. C. and Garling D. J., Minimizing the time required for DNA amplification by efficient heat transfer to small samples,* Anal. Biochem., *186, 328–331 (1990).*

8 *Barany, F., Genetic disease detection and DNA amplification using closed thermostable ligase,* Proc. Natl. Acad. Sci. USA, *88, 189–193 (1991).*

9 *Wallace, R. B., Lin, C-IP., Reyes, A. A., Lowery, J. D. and Ugozzoli L., Ligase chain reaction for the detection of specific DNA sequences and point mutations, in Pfeifer, G. P., ed. Technologies for Detection of DNA Damage and Mutations, New York, Plenum Press, 307–322 (1996).*

10 *Levin, A. J., Momand, J. and Finlay, C. A., The p53 tumor suppressor gene,* Nature, *351, 453–456 (1991).*

11 *Oto, M., Miyake, S. and Yuasa, Y., Optimization of nonradioisotopic single strand conformation polymorphism analysis with a conventional mini-slab gel electrophoresis apparatus,* Anal. Biochem., *213, 19–22 (1993).*

12 *Oto, M., Suehire, T. and Yuasa, Y., Identification of mutated p53 in cancer by non-gel-sieving capillary electrophoretic SSCP analysis,* Clin. Chem., *41, 1787–1788 (1995).*

13 *Pieretti, M., Zhang, R., Fu, Y-H., Warren, S. T., Oostra, B. A., Caskey, C. T.,* et al., *Absence of expression of the FMR-1 gene in fragile X syndrome,* Cell, *66, 817–822 (1991).*

14 *The Huntington's disease collaborative research group, A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes,* Cell, *72, 971–983 (1993).*

15 *Aaltonen, L. A., Peltomaki, P., Leach, F. S., Sistonen, P., Pylkkanen, L., Mecklin, J-P.,* et al., *Clues to the pathogenesis of familial colorectal cancer,* Science, *260, 812–816 (1993).*

16 *Wu, C., Akiyama, Y., Imai, K., Miyake, S., Nagasaki, H., Oto, M., Okabe, S., Iwama, T., Mitamura, K., Masumitsu, H., Nomizu, T., Baba, S., Maruyama, K. and Yuasa Y., DNA alterations in cells from hereditary non-polyposis colorectal cancer patients,* Oncogene, *9, 991–994 (1994).*

17 *Oto, M., Suehiro, T., Akiyama, Y. and Yuasa Y., Microsatellite instability in cancer identified by non-gel sieving capillary electrophoresis,* Clin. Chem., *41, 482–483 (1995).*

18 *Markowitz, S., Wang, J., Myeroff, L., Persons, R., Sun, L. and Lutterbaugh, J., Inactivation of the type II TGF-ß receptor in colon cancers with microsatellite instability,* Science, *268, 1336–1338 (1995).*

19 *Lu, S-L., Akiyama, Y., Nagasaki, H., Saitoh, K. and Yuasa, Y., Mutation of the transforming growth factor-ß type II receptor gene and genomic instability in hereditary non-polyposis colorectal cancer, Biochem. Biophys. Res. Commun., 216, 452–457 (1995).*

20 *Parsons, R., Myeroff, L. L., Liu, B., Willson, J. K. V., Markowitz, S. D. and Kinzler, K. W., Microsatellite instability and mutations of the transforming growth factor-ß type II receptor gene in colorectal cancer,* Cancer Res., *55, 5548–5550 (1995).*

21 *Oto, M., Koguchi, A. and Yuasa, Y., Analysis of a polyadenine tract of the transforming growth factor factor-ß type II receptor gene in colorectal cancers by non-gel sieving capillary electrophoresis,* Clin. Chem., *43, 759–763 (1997).*

Bio-Rad Laboratories

Life Science Group

Website *www.bio-rad.com* ***U.S.*** *(800) 4BIORAD* ***Australia*** *02 9914 2800* ***Austria*** *(01)-877 89 01* ***Belgium*** *09-385 55 11* ***Canada*** *(905) 712-2771* ***China*** *86-10-62051850* ***Denmark*** *45 39 17 99 47* ***Finland*** *358 (0)9 804 2200* ***France*** *(01) 43 90 46 90* ***Germany*** *089 318 84-0* ***Hong Kong*** *852-2789-3300* ***India*** *(91-11) 461-0103* ***Israel*** *03 951 4127* ***Italy*** *02-21609.1* ***Japan*** *03-5811-6270* ***Korea*** *82-2-3473-4460* ***The Netherlands*** *31 318-540666* ***New Zealand*** *64-9-4152280* ***Singapore*** *65-2729877* ***Spain*** *(91) 661 70 85* ***Sweden*** *46 (0)8 627 50 00* ***Switzerland*** *01-809 55 55* ***United Kingdom*** *0800-181134*