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Analysis of polymerase chain reaction products by high-performance liquid chromatography with fluorimetric detection and its application to DNA diagnosis

Hidetoshi Arakawa*, Shuji Nakashiro, Akio Tsuji, Masako Maeda

School of Pharmaceutical Sciences, Showa University, Shinagawa-ku, Tokyo 142, Japan

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Abstract

We describe the development of a sensitive high-performance liquid chromatographic (HPLC) method for polymerase chain reaction (PCR) products using bisbenzimide (Hoechst 33258 dye) based fluorimetric detection. The detection limit and specificity for double-strand DNA detection are improved in comparison with HPLC with UV absorbance detection. This HPLC, using a column packed with diethylaminoethyl-bonded non-porous resin particles, was applied to the detection of allele-specific PCR and restriction fragment length polymorphism analysis. We also developed a hybridization method analyzed by HPLC. DNA fragments (149 bp) containing the mutation site (C→A,G,T) in the N-ras gene were amplified by PCR. Fluorescein isothiocyanate (FITC)-labeled DNA probes were also prepared by PCR using FITC-labeled 5′ primer. Analysis of mutation was performed by the separation of a hybrid and non-reactive DNA probe with HPLC with fluorimetric detection after the hybridization of target DNA (149 bp) and a FITC DNA probe. The effects of various factors on hybridization were examined to establish optimal assay conditions. Under the conditions determined, a point mutation in PCR products obtained from the N-ras gene could be detected specifically by this method. The analysis of PCR products by HPLC may potentially be useful for DNA diagnosis.

Keywords: Polymerase chain reaction; DNA; Hybridization

1. Introduction

DNA analysis is now being widely used in genetic research, the diagnosis of genetic and infectious diseases, forensic sciences, plant breeding and other fields. The polymerase chain reaction (PCR) procedure has been used to amplify a specific segment of a target gene enzymatically, which produces a 10^5-fold increase in the amount of target sequences [1]. Detection of the PCR product is accomplished by dot blot hybridization assay, the method of ethidium staining-gel electrophoresis or Southern blot hybridization, which is carried out by hybridization with a DNA probe followed by slab gel electrophoresis. These detection methods are time-consuming and complicated and therefore are not well suited for clinical laboratories. Recently, capillary electro-

*Corresponding author.

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phoresis (CE) [2] and high-performance liquid chromatography (HPLC) [3] have been used to detect PCR products. Since both methods can detect PCR products on-line, full automation of a PCR detection system is feasible. It is well known that the resolving power of CE is greater than that of HPLC; for example, it is possible to separate a few base pairs of double-strand DNA and the secondary conformation of single-strand DNA, such that the high separation efficiency of CE is utilized for the analysis of polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and PCR-single-strand DNA conformation polymorphism [3]. However, CE instruments with laser-induced fluorescence detection are not yet widely available in clinical laboratories. Kato et al. described a highly efficient and rapid separation of typical DNA restriction fragments using a column packed with diethylaminoethyl-bonded non-porous resin particles (DEAE-NPR) [3]. Katz and Haff reported a rapid separation and purification of PCR products by HPLC using the DEAE-NPR column [4]. Recently, Asakawa et al. reported a detection method for heterozygous carriers of a deletion by combined PCR-RFLP and HPLC using the column described above [5].

In this paper, we describe the development of a sensitive HPLC method for PCR products using bisbenzimide (Hoechst 33258 dye) based fluorescence detection. The intercalation of dye to double-strand (ds) DNA was performed on-column. This method improved the detection limit and specificity for ds DNA detection in comparison with HPLC with UV absorbance detection. This HPLC method with fluorimetric detection was applied to the detection of allele-specific PCR of phenylketonurea (PKU) and RFLP analysis of the dive E gene. We further studied the possibility of using HPLC for analysis of DNA hybridization: sample DNA amplified with PCR was hybridized with a fluorescein isothiocyanate (FITC)-labelled DNA probe in a tube, and the hybrid and non-reacted FITC-labelled DNA probe was separated by HPLC with fluorimetric detection. This method can identify the nucleotide sequence of a point mutation in a PCR product and is more suitable than the conventional hybridization method used for diagnostic genetics and infectious diseases.

2. Experimental

2.1. Apparatus

The Guliver® HPLC system (Jasco, Tokyo, Japan) was used. Fluorimetric detection of separation was carried out with 821-FPS (Jasco), and laser-induced fluorescence detection was carried out with LF-8010 (Toso, Tokyo, Japan).

2.2. Materials

The DEAE-NPR column was purchased from Tosoh (Tokyo, Japan). ϕx174RF DNA/Hae III digest (DNA MW Marker 4) was obtained from Nippon Gene (Osaka, Japan). Bisbenzimide (Hoechst 33258) was purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). The gene Amp® PCR reagent kit with Amplitaq® DNA polymerase was from Perkin-Elmer Cetus (Norwalk, CT, USA). PKU1, PKU15W and PKU16M for the hepatic phenylalanine hydroxylase gene (PAH) and dried blood spots of phenylketonurea with the $Arg^{413} \rightarrow Pro^{413}$ mutation site of exon 12 were generously donated by Professor Narizawa (Tohoku University Medical School, Miyagi, Japan), and primer 1, primer 2 and the dive E gene were generously donated by Professor Ohki (Kyourin University, Tokyo, Japan). Hae III was purchased from Toyobo (Osaka, Japan). Human DNA was generously donated by Dr. Tobe and Professor Tomita (Showa University, Department of Biochemistry, Tokyo, Japan). FITC-labelled primer and the various primers used in this experiment were synthesized by Takara Shuzo (Osaka, Japan).

2.3. Methods

2.3.1. Allele-specific PCR of phenylketonurea

This process was carried out according to the method of Narisawa and Matsubara [6]. DNA was extracted from dried blood specimens on Guthrie cards that are generally used for neonatal screening. Three discs (3 mm diameter) containing about 10.8 μl of blood were punched from a specimen by a steel punch and placed in a 1.5 ml polypropylene tube.

The discs were fixed in 30 μl methanol for 5 min at room temperature, dried under vacuum, and incubated in 90 μl of solution containing 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 0.001% gelatin and 50 μg/ml proteinase K at 55°C for 1 h. The mixture was then heated at 99°C for 10 min and centrifuged at 15 000 *g* for 10 min. Ten microliters of the supernatant (extracted DNA) was subjected to PCR using PKU1 and PKU15W primer and PKU1 and PKU16M primer. The PCR reaction mixture consisted of 50 mmol/l KCl 10 mmol/l Tris–HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 0.001% gelatin, 200 μmol/l dNTPs, 1 μmol/l each of the amplification primers, 10% dimethyl sulfoxide, 10 μl extracted DNA, and 1.5 units *Taq* DNA polymerase in a total volume of 30 μl. A DNA thermal cycler (ATTO, Zymoreactor, Tokyo, Japan) was used to carry out 30 cycles of PCR under the following program: 1 min denaturation at 94°C, 1 min annealing at 56°C and 1 min extension at 74°C. Ten microliters of the PCR product was analyzed by the following HPLC.

2.3.2. PCR-RFLP

This experiment was carried out using the dive E 42 gene and by the procedure described previously [7,8]. The method is briefly as follows: the PCR product obtained using primer 1 and primer 2 was digested with Hae III. The sample was then analyzed under the following HPLC conditions.

2.3.3. HPLC conditions for the analysis of PCR products

The column was a TSK gel DEAE-NPR (35×4.6 mm I.D.) (Tosoh). DNA fragments were separated by gradient elution of sodium chloride (0.5 to 1 mol/l for 30 min) in 20 mmol/l Tris–HCl (pH 8.0) containing 100 ng/ml bisbenzimide. The fluorescence of the DNA fragment was detected at 455 nm with excitation at 350 nm.

2.3.4. Preparation of samples containing a point mutation

Samples of N-ras genes containing wild-type ($C^{181}AA$) and mutation-type ($A^{181}AA$, $G^{181}AA$ and $T^{181}AA$) in the N-ras gene (at 61 codone) were used as the model DNA. A wild and three mutant DNA fragments (149 bp) of the N-ras gene were prepared by PCR using wild primer (primer 61-C) and mutants primer (primer 61-A, primer 61-G and primer 61-T) (Table 1a) from 1 mg of normal human DNA. The obtained PCR products (wild and three kinds of point mutant) were purified using Centricon® to remove PCR reagents.

2.3.5. Preparation of FITC-labelled DNA probe

DNA probes (69, 95, 117 bp) labelled with FITC were also prepared by PCRs using primer FITC as a sense primer and primer 3′-69, primer 3′-95 and primer 3′-117 as an anti-sense primer (Table 1b). The templates of wild and mutants of the N-ras gene

Table 1
Nucleotide sequence of the primers used

	Sequence (5′→3′)	Position
(a)		
Primer 61-C (sense for wild)	GGTGAAACCTGTTTGTTGGACATACTGGATACAGCTGGACAAG	142–184
Primer 61-A (sense for mutant)	GGTGAAACCTGTTTGTTGGACATACTGGATACAGCTGGAAAAG	142–184
Primer 61-G (sense for mutant)	GGTGAAACCTGTTTGTTGGACATACTGGATACAGCTGGAGAAG	142–184
Primer 61-T (sense for mutant)	GGTGAAACCTGTTTGTTGGACATACTGGATACAGCTGGATAAG	142–184
Primer 3′-149 (anti-sense)	GTGTAGAGGTTAATATCCGC	271–290
(b)		
Primer FITC (sense)	FITC-GGTGAAACCTGTTTGTTG	191–210
Primer 3′-69 (anti-sense)	TTGGTCTCTCATGGCACTGT	217–236
Primer 3′-95 (anti-sense)	AGGAAGCCTTCGCCTGTCCT	241–258
Primer 3′-117 (anti-sense)	ATTATTGATGGCAAATAC	142–159

were PCR products which were prepared as samples containing a point mutation.

2.3.6. Hybridization assay

A 6.25 μl volume of wild or mutant DNA sample (149 bp) and 6.25 μl of the FITC-labelled DNA probe (95 bp) was added to 12.5 μl hybridization buffer containing 1 mol/l NaCl; the reaction mixture was heat-denatured at 99°C for 5 min, cooled in ice for 5 min, then incubated for hybridization (annealing) at 47 or 52°C for 30 min. Ten microliters of the hybridization reaction mixture was analyzed by HPLC with fluorimetric detection (HPLC–FL).

2.3.7. HPLC for the detection of hybridization

The separation conditions were similar to those used for the PCR products, except for the use of eluents without bisbenzimide. Fluorimetric detection of the FITC-labelled DNA probe and its hybrid was performed with excitation at 490 nm and emission at 520 nm.

3. Results and discussion

3.1. Separation of ϕx174RF DNA/Hae III digest by HPLC using fluorimetric detection

Kato et al. [3] previously reported rapid separation of DNA restriction fragments using a column packed with DEAE-NPR. In order to obtain a higher detection limit for PCR products (double-strand DNA fragment), we studied HPLC–FL with a DEAE-NPR column. Ethidium bromide (EB), bisbenzimide [9,10] and DAPI [11] are generally used as fluorescence dyes (FL dye) for the detection of ds DNA. EB, which is a powerful mutagen and biohazard, is not adequate as an FL dye used for a routine assay. Therefore, bisbenzimide and DAPI were studied as FL dyes for HPLC of DNA. The results of DNA detection obtained using both dyes showed almost the same sensitivity. Therefore, fluorescence bisbenzimide was chosen as the FL dye in the following experiments. To develop a simple and rapid method for DNA determination, we studied the on-column derivatization method in which the intercalation of dye and separation of DNA are performed simultaneously. For the on-column derivatization system, it is preferable that the dye is contained in elution buffer. The optimal concentration of the dye was determined as a compromise between the sensitivity of the DNA fragment and the baseline level on the chromatogram. When a dye concentration of more than 100 ng/ml is used, both the fluorescence intensity signal resulting from the intercalation of the dye into DNA, and the baseline, increased. As the dye concentration decreased, the sensitivity of DNA detection also decreased. Therefore, 100 ng/ml Hoechst dye was chosen as the optimal concentration. The optimal separation conditions of ϕx174RF DNA/Hae III digest were determined as described in the Methods section. The chromatogram of the ϕx174RF DNA/Hae III digest is shown in Fig. 1. The separation of DNA fragments (72, 118, 194, 234, 271/281/310, 603, 872, 1078 and 1310 bp) was achieved in 15 min. These peaks were identified by spiking with each DNA fragment separated by agarose electrophoresis. The elution pattern obtained by this fluorimetric detection was slightly different from that by UV absorbance detection (data not shown). The fluorescence intensity produced by a complex of dye and DNA fragment was not completely proportional to the size of the DNA fragment, for example the fluorescence intensity of 118 bp is higher than that of 194 bp. This conflict resulted from the difference in the fluorescence enhancement, which is two times greater upon interaction with the A–T pair compared to the G–C pair [10]. However, the resolution of separation and the order of elution of the DNA fragments were not affected by intercalation of the dye to the DNA fragment. Theoretical plate numbers of the present method are 16 700 for 194 bp and 31 800 for 1353 bp. These plate numbers are approximately the same as that obtained by HPLC using UV absorbance detection (HPLC–UV) without FL dye (data not shown). Therefore, bisbenzimide was found not to be effective for the separation of double-strand DNA fragments.

The sensitivity of this HPLC–FL for DNA was compared with that obtained by UV absorbance detection. This method, using bisbenzeimide as FL dye, is about four times as sensitive as with UV absorbance detection. The detection limit of the 1078 bp fragment was 2.2 fmol ($S/N=5$). Furthermore, this HPLC–FL is also more specific for double-

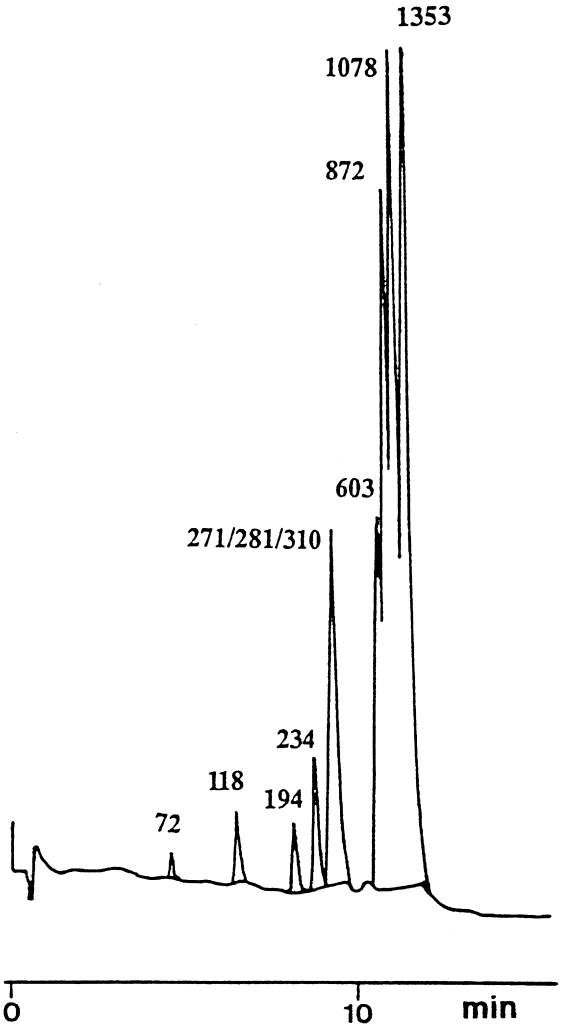

Fig. 1. Chromatogram of the Hae III digest of ϕx174RF DNA.

strand DNA than HPLC with UV absorbance detection. The specificity of this method can be shown by the analysis of PCR products. The chromatograms of the PCR product of the λ-phage obtained by UV absorbance and fluorimetric detection are shown in Fig. 2. As shown in Fig. 2, a 500 bp fragment of the PCR product was eluted at 10 min, and peaks of dNTP and primers, which are PCR reagents, are not present in the chromatogram obtained by fluorimetric detection. The coefficient of variation (CV%, $n=7$) of the elution time and the peak hight of the PCR-amplified 500 bp fragment were 0.6 and 2.73%, respectively. The detection limit of the λ-phage was

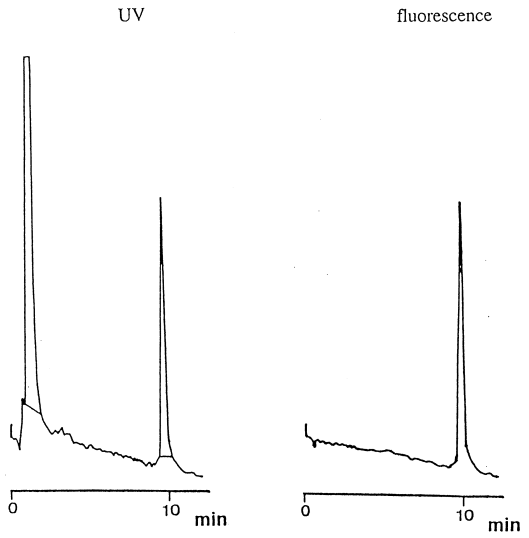

Fig. 2. Chromatograms of the PCR product of λ-phage obtained by HPLC with UV absorbance and fluorescence detection. Ten picograms of λ-phage was used as a template of PCR. The PCR product diluted 10-fold with water was used for separation with fluorescence detection.

100 fg as the PCR template. This sensitivity is higher than that of slab gel electrophoresis with ethidium bromide as fluorescence dye.

3.2. Allele-specific PCR of phenylketonurea

Allele-specific PCR is a method for the determination of a point mutation in a gene [12]. This method depends on whether the PCR product is observed in slab gel electrophoresis following PCR with a primer-specific mutation site. The basis of this system is that oligonucleotides with a mismatched 3′-residue do not function on DNA polymerase reaction. Matsubara et al. [13] have developed an allele-specific PCR for the phenylketonurea (PKU) mutation. We tried to apply HPLC analysis to the detection of this allele-specific PCR technique for PKU. As a model, the PCR product obtained by allele-specific PCR of $Arg^{413}(CGC)\rightarrow Pro^{413}(CCC)$ of exon 12 in the hepatic phenylamine hydroxylase (PAH) gene was detected by HPLC–FL. DNA was extracted from dried blood specimens on Guthrie Cards according to the method of Matsubara et al. [13]. Extracted DNA was amplified separately using PKU1 and PKU15W primer, and PKU1 and

PKU16M primer. PKU15W primer corresponds to the normal sequence at Arg^{413}(CGC) and PKU16M primer corresponds to the point mutation sequence at Pro^{413}(CCC). For the normal subject, the product (138 bp) was derived only from PKU1 and PKU15W, but the product was not generated when the PKU16M primer was applied to normal DNA. For the patient sample containing a point mutation at Pro^{413}, no product was generated by PKU1 and PKU15W primer, whereas the product was observed when PKU15W primer was replaced by PKU16M primer. The PCR products obtained were analyzed by HPLC–FL. As shown in Fig. 3, the PCR product was eluted nearly 7 min. Comparison of these results will indicate whether or not the DNA sample contains a point mutation in exon 12. The chromatograms show that a non-specific peak was observed at 1–2 min, which is due to unknown materials from the blood sample.

3.3. PCR-RFLP

PCR-RFLP is also a specific method for determining a single base mutation in genes. In addition, this method has been used to diagnose many diseases. In

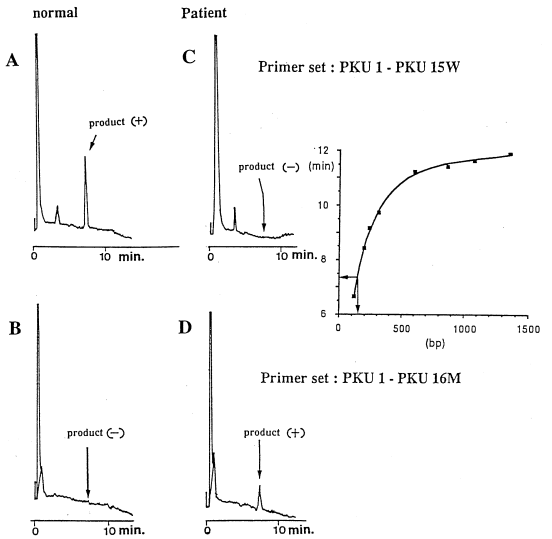

Fig. 3. Chromatograms of the products obtained by allele-specific PCR. (A,B) Results for the normal subject with primer set PKU1–PKU15W and PKU1–PKU16M, respectively. (C,D) Results for PKU subject with primer set PKU1–PKU15W and PKU1–PKU16M, respectively..

the present study, we tried to apply HPLC–FL to the detection of PCR-RFLP. The model used the dive E 42 gene carrying wild- and mutant-type DNA. The mutant type of this DNA contains a G→A point mutation at the 141 site. Wild and mutant DNA were subjected to PCR to amplify the 359 bp fragment in the dive E 42 gene. This region contains the mutation site of $G^{141}\rightarrow A^{141}$. The two respective PCR products generated from the wild and mutant gene are the ^{140}GGCC and ^{140}GACC sites in the 359 bp fragment. The restriction enzyme, Hae III, cuts the GGCC site in the PCR product generated from a wild gene, whereas the PCR product of a mutant is not cut by this enzyme. Thus, this Hae III recognition site is lost in mutated DNA. The PCR reaction was carried out according to the method of Tamura et al. [7] and Kawamata and Ouki [8] as described in the Methods section. The amplification product was digested directly by the restriction enzyme, Hae III, for 3 h at 37°C, and the sample was subsequently analyzed by HPLC–FL. As predicted from the dive E sequence, the Hae III restriction enzyme cut the GGCC site in the PCR product of wild genes and produced two peaks (122 bp) at 6 min and (237 bp) 8 min. In contrast, the PCR product of a mutant gene, digested by Hae III, showed only one peak (359 bp) at 9 min. The results are shown in Fig. 4. The chromatograms show that HPLC–FL analysis for PCR-RFLP is a specific and sensitive method for ds DNA digested with a restriction enzyme.

3.4. Detection of a hybridization assay by HPLC

The Southern-HPLC method was developed to detect a point mutation in the N-ras gene. Although PCR itself is a specific method, Southern blot hybridization is generally used in order to identify the nucleotide sequence of a PCR product exactly. The process involves a separation of DNA amplified by PCR with gel electrophoresis, transfer to a membrane support and hybridization with a labelled probe. Although this method is specific and sensitive, it is time-consuming and laborious. In this study, we investigated the analysis of a hybridization product by HPLC.

It is well known that the efficiency and specificity of hybridization are basically affected by the base size of the probe used, annealing temperature, con-

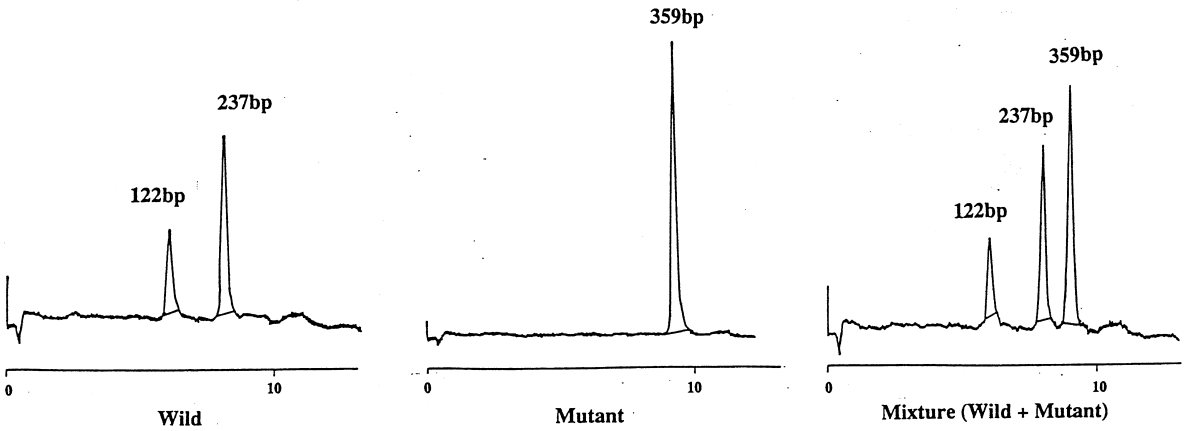

Fig. 4. Chromatograms of an Hae III restriction digest of PCR products obtained from wild and mutant dive E gene.

centration of salt and incubation time. As a preliminary experiment, we studied these effects using DNA fragments (149 bp) containing various point mutations (C, A, G, T) at 61 codon of the N-ras gene as a model DNA sample. These DNA fragments (149 bp) of the N-ras gene were prepared by PCR from 1 μg of human DNA according to the procedure in the Methods section. The nucleotide sequences used are shown in Table 1a. DNA probes (69, 95, 117 bp) of the wild-type (sequence C) at 61 codon labelled with FITC, which is of a different base size, were also prepared by PCR using FITC-labelled 5′-primer and primer 3′-69, primer 3′-95 and primer 3′-117, respectively. These nucleotide sequences are shown in Table 1b. Hybridization was performed in a micro tube (0.5 ml) containing HEPES buffer (pH 8.0) as follows: PCR product 149 bp (sample) of wild-type or mutant and/or PCR product 500 bp of λ-phage DNA were mixed with a FITC-DNA probe 95 bp of wild-type and incubated at optimal annealing temperature. The effect of various conditions (annealing temperature, salt concentration, reaction time, nucleotide length (base size) of FITC-DNA probe) on the hybridization efficiency was studied. The efficiency was evaluated from the peak hight of the hybrid which was eluted in about 12 min. In a study of NaCl concentration, the hybridization was performed in 100 mmol/l HEPES buffer containing 0–2 mol/l NaCl. The efficiency of the hybrid approached a plateau with 0.5 mol/l NaCl. Therefore, 1 mol/l NaCl was chosen for the following experiments. The effect of reaction time on the hybridization (annealing) was also studied; 30 min showed a high yield of hybridization. Both the reaction temperature and the base size of the FITC-DNA probe are important factors for specificity of the hybridization. Therefore, the effects of these two conditions on specificity of the hybridization were studied using FITC-DNA probes of differing base size at various annealing temperatures. Table 2 shows the results for specificity on the hybridization obtained with different DNA probe lengths of 69 (A), 95 (B) and 117 (C) bp.

The results show that when an FITC-DNA probe of 69 bp (wild-type) was used, an annealing temperature of 50°C showed high specificity against the N-ras gene containing a point mutation (A, G and T) and λ-phage DNA, but the hybridization yield was low. When an FITC-DNA probe of 117 bp (wild-type) was used, the hybridization yield was the highest of these three probes, however the specificity was less against mutation $T^{181}AA$. Therefore, a FITC-DNA probe of 95 bp was used in the following experiments. Using a FITC-DNA probe of 95 bp, a high specificity and a reproducible peak height of the hybrid could be obtained at 47°C annealing temperature. Under these conditions, this method clearly distinguished wild and mutants of a point mutation in the PCR products obtained from the N-ras gene.

Fig. 5 shows a chromatogram obtained from the hybridization of human DNA and a FITC-DNA probe (95 bp, wild-type). The first peak at 8 min

Table 2
Effect of annealing temperature on the specificity of hybridization (%) using FITC-labeled probes of various base size

Target DNA	30°C	37°C	40°C	42°C	45°C	50°C
(A) *By probe length of 69 bp*						
Normal C	100	100	100	100	100	100
Mutation A	80	80	64	50	45	0
Mutation G	84	80	57	50	45	0
Mutation T	80	80	57	50	22	0
λ-Phage	0	0	0	0	0	0

Target DNA	30°C	40°C	45°C	47°C	50°C
(B) *By probe length of 95 bp*					
Normal C	100	100	100	100	0
Mutation A	95	83	50	17	0
Mutation G	95	91	50	17	0
Mutation T	74	74	50	17	0
λ-Phage	11	17	6	17	0

Target DNA	30°C	40°C	45°C	50°C	52°C	55°C
(C) *By probe length of 117 bp*						
Normal C	100	100	100	100	100	100
Mutation A	79	79	77	57	40	33
Mutation G	79	79	77	29	50	33
Mutation T	107	85	100	86	90	100
λ-Phage	0	5	8	0	20	0

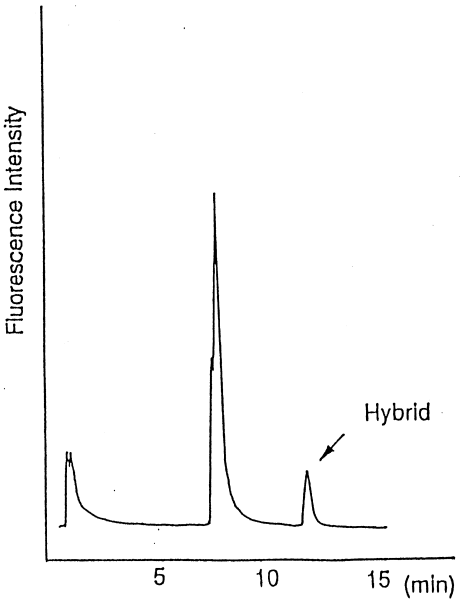

Fig. 5. Chromatogram obtained from hybridization of human DNA (wild-type) and a FITC-DNA probe (wild-type).

shows a non-reactive FITC-DNA probe and the second peak at 12 min is a hybrid of a sample (149b base) and an FITC-DNA probe (95 base). The coefficient of variation (CV%) of the elution time and the peak height of the hybrid were 0.17 and 3.73% for within-assay ($n=9$) and 0.45 and 3.46% for day-to-day ($n=8$).

In addition, we prepared a FITC-DNA probe (95 base size) of three mutants (A, G and T) to identify a point mutation in the PCR product. In this experiment, the annealing temperature was increased to 53°C and 1 μl dimethyl sulfoxide (DMSO) was added to the hybridization reaction mixture to obtain a highly stringent hybrid. The increase in annealing temperature and the addition of DMSO eliminated non-specific hybridization and also resulted in reduction of the hybrid yield. Therefore, a more sensitive method, such as laser-induced fluorescence detection, was required to detect the small signal of a specific hybrid.

DNA samples containing wild and a point mutation were hybridized with four kinds (probe A:

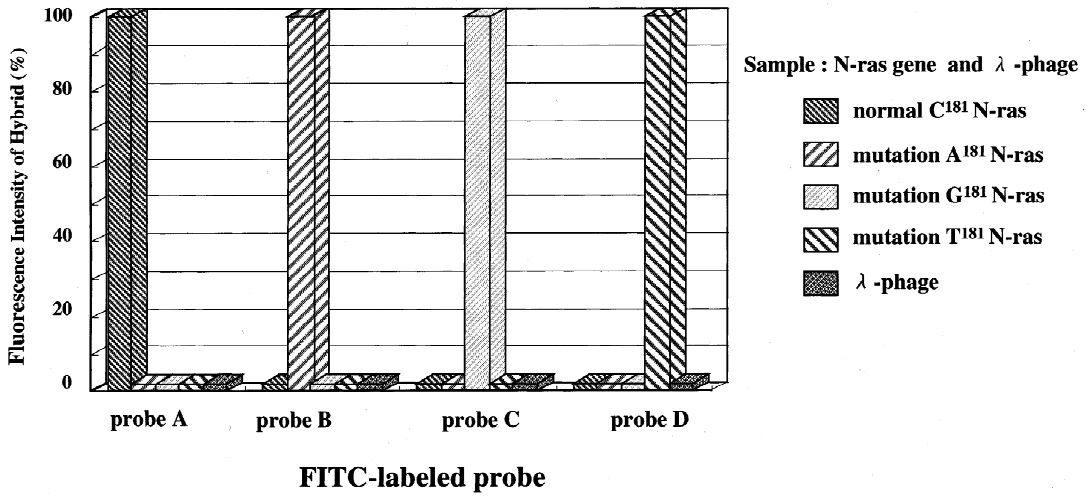

Fig. 6. Specificity of four different FITC-labelled 95 bp probes for the detection of a point mutation in 149 bp PCR products (probe A: normal C^{181}, probe B: mutant A^{181}, probe C: mutant G^{181}, probe D: mutant T^{181}).

normal C^{181}, probe B: mutant A^{181}, probe C: mutant G^{181} and probe D: mutant T^{181}) of FITC-DNA probes and were analyzed by HPLC with a laser-induced fluorescence detection method. The results are shown in Fig. 6. Only a perfect match, in which the nucleotide sequence of the DNA sample was completely consistent with the FITC-DNA probe used, showed a high peak of the hybrid, whereas mismatch hybridization did not show a peak. This result shows that this method is highly specific against a nucleotide sequence of a PCR product and can be used to identify the composition of the base of a point mutation in a PCR product.

4. Conclusion

Analysis of PCR products by HPLC with fluorometric detection is specific and sensitive for ds DNA and can be used for the analysis of allele-specific PCR and PCR-RFLP. Furthermore, the combination of HPLC and the hybridization technique is more rapid, simpler and more reproducible than the Southern hybridization method carried out by gel electrophoresis and blotting to the membrane. Therefore, these methods are very useful for the analysis of PCR products for diagnostic genetic and infectious diseases.

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References

[1] R.K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, N. Arnheim, Science 230 (1985) 1350.
[2] Y. Baba, J. Chromatogr. B 687 (1996) 271–302.
[3] Y. Kato, Y. Yamasaki, A. Onaka, T. Kitamura, T. Hashimoto, J. Chromatogr. 478 (1989) 264–268.
[4] E.D. Katz, L.A. Haff, J. Chromatogr. 512 (1990) 433–444.
[5] J. Asakawa, C. Satoh, Y. Yamasaki, S. Chen, Proc. Natl. Acad. Sci. USA 89 (1992) 9126.
[6] K. Narisawa, Y. Matsubara (in preparation).

[7] F. Tamura, S. Nishimura, M. Ohki, EMBO J. 3 (1984) 1103.
[8] S. Kawamata, R. Ohki, Kyorin Igaku Kaishi 24 (1993) 111.
[9] T.R. Downs, W.W. Wilfinger, Anal. Biochem. 131 (1983) 538.
[10] W. Sterzel, P. Bedford, G. Eisenbrand, Anal. Biochem. 147 (1985) 462.
[11] M. Legros, A. Kepes, Anal. Biochem 147 (1985) 497.
[12] C.R. Newton, A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith, A.F. Markham, Nucleic Acids Res. 17 (1989) 2503.
[13] Y. Matsubara, K. Narisawa, K. Tada, H. Ikeda, Y. Yegi, D.M. Danks, A. Green, R.B. McCabe, Lancet 371 (1991) 552.