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A simple method for detecting single base substitutions and its application to HLA-DPB1 typing

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ABSTRACT

We have developed a simple and reliable method, PCR-PHFA (polymerase chain reaction dependent preferential homoduplex formation assay), for detection of single base substitutions within PCR amplicons. This technique is based upon strand competition during hybridization between a double labeled amplicon, prepared from biotin and DNP labeled primers, and an unlabeled amplicon. Under the precisely controlled temperature gradient, the preferential formation of a homoduplex over a heteroduplex occurs. After annealing, the identical sequence of the double labeled and unlabeled amplicon resulted in a low population of regenerated double labeled dsDNA due to strand exchange between them. Even when the two differed by only a single base substitution, double labeled molecule was regenerated efficiently because of preferential homoduplex formation. The regenerated double labeled molecule was captured onto a streptavidin coated microtiter plate and quantified enzymatically with a chromogenic substrate. The technique has been successfully applied in HLA-DPB1 typing. Furthermore, we detected a mutated gene even in the presence of a large excess of the corresponding normal gene.

INTRODUCTION

Recent advances in DNA technology has enabled us to define the nature of mutations that causes inherited diseases. The most powerful tool in this field has been the polymerase chain reaction (PCR) (1). Some causes of inherited diseases have been identified as a single base substitution at the given locus. The techniques such as sequence specific oligonucleotide probe (SSO) (2), sequence specific primer (SSP) (3), and ligation reaction (4) can identify known single base substitutions. However, these are inadequate in detecting 'unknown' substitutions. In order to overcome this limitation, several techniques have been devised; including single strand conformation polymorphism (SSCP) (5), denaturing gradient gel electrophoresis (DGGE) (6,7), and ribonuclease cleavage at mismatches in DNA:RNA duplexes (8). However, these methods are too complicated for routine DNA diagnosis. Development of a simple and reliable method in order to differentiate a mutant allele from the normal gene when the two differ by only a single substitution at an unknown position, is of fundamental importance in DNA diagnosis and molecular genetics (9).

Terouanne *et al.* reported an elegant technique based on DNA strand competition during hybridization, to detect 'unknown' mutations in a PCR amplicon (10). In their method, the biotin and FITC double labeled amplicon (served as probe) and an excess of unlabeled amplicon (served as sample) were hybridized in solution under the precisely controlled temperature gradient. When the sequences of a labeled amplicon and an unlabeled amplicon were identical, the population of double labeled dsDNA decreased due to mathematical dilution with an unlabeled amplicon. On the other hand, when a labeled amplicon differed even by a single base, each ssDNA in the denatured mixture formed a duplex preferentially with a completely matched ssDNA. As a result, the original double labeled dsDNA was not diluted with unlabeled amplicon. The regenerated double labeled dsDNA was quantified by means of bioluminescent assay. As elegant as it is, this method still has limitations for routine DNA diagnosis: the detection system is complicated and requires expensive apparatus, and it is difficult to detect mutations in heterozygotes, because a half of an excess amount of unlabeled amplicon is identical to a labeled amplicon.

To overcome these problems, we have established PCR-PHFA (polymerase chain reaction dependent preferential homoduplex formation assay), in which we used a simple means of detection, ED-PCR (enzymatic detection of PCR product) (11,12), to quantify the double labeled dsDNA. Furthermore, we have refined the system so that it can detect a mutant allele in the presence of an excess of the normal gene.

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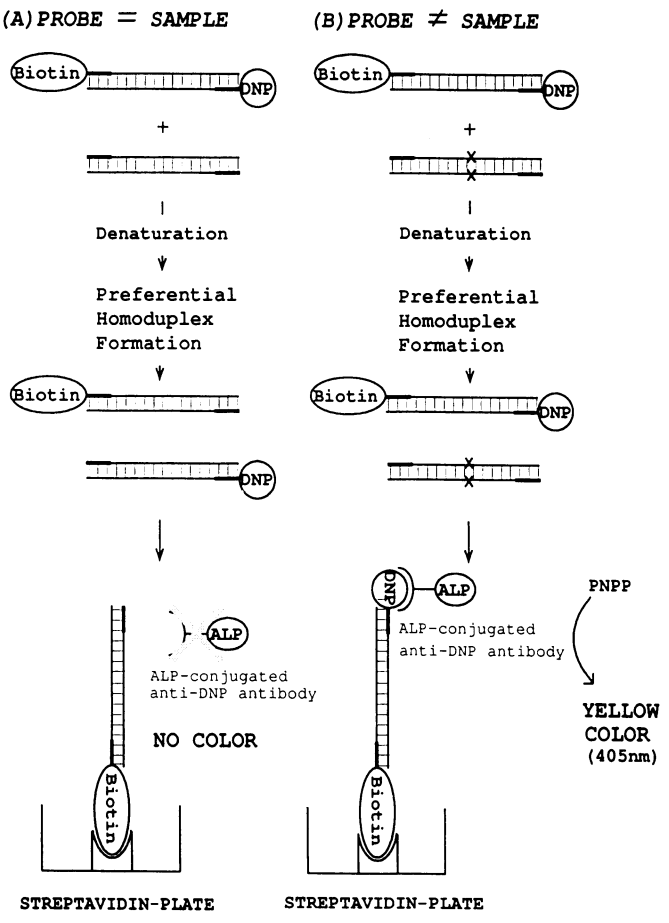

Figure 1. Principle of PCR-PHFA. Double labeled PCR amplicons were prepared with biotin and DNP labeled primers. Unlabeled amplicons were prepared with aminoalkylated primers. Labeled and unlabeled amplicons were mixed and denatured at 98°C for 10 min, then cooled slowly to 70°C at the rate of 1°C/10 min. (**A**): When labeled and unlabeled amplicons were identical, the population of double labeled dsDNA decreased due to dilution with the unlabeled molecule. (**B**): When two amplicons differed by even a single base, preferential homoduplex formation occurred. As a result, the population of double labeled dsDNA did not decrease drastically. Double labeled dsDNA was captured on streptavidin coated microtiter well via biotin/streptavidin affinity, and quantified with alkaline phosphatase conjugated anti DNP antibody and PNPP as a chromogenic substrate.

MATERIALS AND METHODS

Oligonucleotides and labeled primers

Oligonucleotide primers were synthesized on an Applied Biosystems model 381A DNA synthesizer, and an amino group was added to the 5′ terminus using Aminolink2. These aminoalkylated oligonucleotides were used as unlabeled primers. Biotin and DNP (Dinitrophenyl) groups were introduced to aminoalkylated oligonucleotides using CAB-NHS ester (BRL) and 2,4-dinitrofluorobenzene, respectively. Purification of labeled primers proceeded by means of HPLC on a C18 reversed phase column (μBondapak C18).

DNA sources

The plasmid pBR322-HbS was provided by K.Itakura (Beckman Res. Inst. of City of Hope, Duarte, CA). HLA-DPB1 DNAs were prepared by using PCR and cloned into the pSP64 vector. The plasmids pSK-2 (13) and pKY-2 (14) were purchased from the Japan Cancer Research Resources Bank. Human genomic DNA was prepared as described previously (15).

PCR amplification

PCR amplifications were performed using T*th* DNA polymerase in a 100 μl reaction mixture as recommended by the manufacturer, in a Thermal Cycler PJ-2000 (Perkin Elmer). The amplification cycle consisted of denaturation at 94°C for 5 min followed by 30 cycles of denaturation (94°C, 30 sec), annealing (50°C, 30 sec), and extension (72°C, 1 min). The double labeled PCR amplicon (see Fig.1) was prepared with biotin-labeled and DNP-labeled primers, and an unlabeled amplicon was prepared with aminoalkylated primers. The amplicons were used for hybridization without purification.

Hybridization

Hybridization was performed in 36 μl of a reaction mixture containing 1μl of double labeled amplicon, 15 to 20 μl of unlabeled amplicon, and 6 μl of 20×SSC. The reactions proceeded in 500 μl Eppendorf tubes, in which the mixtures were overlaid with light mineral oil, on a Thermal Cycler PJ-2000 (Perkin Elmer). For HLA-DPB1 typing, the reactions were performed in Falcon 3911 microplate with Programmable Thermal Controller (M.J.Research Inc.). For hybridization with temperature gradient, the samples were denatured at 98°C for 10 min, then slowly cooled to 70°C at a rate of 1°C per 10 min.

Detection of the double labeled molecule

Hybridization mixture, 25 μl, was transferred to a streptavidin-coated microtiter well with 100 μl of anti-DNP antibody conjugated alkaline phosphatase in solution I: 50 mM Tris−HCl pH 7.5, 150 mM NaCl, and 0.05% Tween 20. After incubation for 30 min at 25°C, the mixture was aspirated and the well was washed twice with 350 μl of solution I. Thereafter 100 μl of chromogenic substrate solution, 40 μg/ml PNPP (*p*-nitro phenyl phosphate), 1 M Diethanolamine buffer pH 9.8, 0.5 mM $MgCl_2$, was added to each well. The coloring reaction proceeded at 25°C for 30 or 60 min and the absorbance at 405 nm was read using a microtiter plate reader.

RESULTS AND DISCUSSION

PCR-PHFA

Terouanne *et al.* reported a novel method of distinguishing single base substitutions at unspecified sites in PCR amplicons (10). They used a bioluminescent assay, which does not require any separation to detect the double labeled dsDNA. Although it is an excellent assay, many kinds of reagents and an expensive luminometer are needed. In order to simplify the detection method, we applied our ED-PCR system (11,12) for the detection of biotin and DNP labeled dsDNA and named PCR-PHFA (polymerase chain reaction dependent preferential homoduplex formation assay) (Fig. 1). In this system, the double labeled PCR amplicon and an excess of unlabeled amplicon are hybridized in a solution with a temperature gradient. DNA strand competition occurs and a specific homoduplex formation takes place. This preferential homoduplex formation is specific enough to distinguish a single base substitution. The double labeled molecule is captured on streptavidin coated microtiter well via streptavidin/biotin affinity and detected by anti-DNP antibody conjugated alkaline phosphatase using PNPP as a chromogenic

Table 1. Nucleotide sequences of oligonucleotides

PG1	(Bio or Amino)	5'ACACAACTGTGTTCACTAGC
PBG1	(Bio or Amino)	5'GGGTTGGCCAATCTACTCCCAG
PG2	(DNP or Amino)	5'CAACTTCATCCACGTTCACC
PBG2	(DNP or Amino)	5'TGGTCTCCTTAAACCTGTCTTG
PBG3	(DNP or Amino)	5'CAGCCTAAGGGTGGGAAAAT
PBG4	(DNP or Amino)	5'CTTCACCTTAGGGTTGCCCA
PBG5	(DNP or Amino)	5'AGCTCACTCAGTGTGGCAAA
DPBAMP-A	(Bio or Amino)	5'CCTCTCCGCAGAGAATTAC
DPBAMP-B	(Bio or Amino)	5'AGCCCTCACTCACCTCGGCG
PHR-1	(Bio or Amino)	5'ATGACGGAATATAAGCTGCT
PHR-3	(Bio or Amino)	5'CTGGATGGTCAGCGCACTCTT

Biotin and DNP labeled oligonucleotide primers were used to prepare labeled amplicons. Aminoalkylated oligonucleotide primers were used to prepare unlabeled amplicons.

Table 2. The effect of combinations of labeled and unlabeled amplicon for length on the detection of single base substitution by PCR-PHFA

Labeled amplicon	Unlabeled amplicon PG1/PG2		PG1/PG2		PBG1/PG2		PBG1/PBG2	
(A) 98°C to 70°C, 1°C/10 min								
normal	N	M	N	M	N	M	N	M
PG1/PG2(109bp)	**0.04**	**0.39**	0.36	0.60	0.44	0.64	0.54	0.61
PG1/PBG2(166bp)	0.73	0.73	**0.05**	**0.32**	0.72	0.79	0.60	0.72
PBG1/PG2(205bp)	0.78	0.77	0.78	0.76	**0.04**	**0.30**	0.41	0.56
PBG1/PBG2(262bp)	0.83	0.80	0.80	0.79	0.78	0.78	**0.06**	**0.38**
(B) 98°C to 40°C, in 1 min								
normal	N	M	N	M	N	M	N	M
PG1/PG2(109bp)	**0.05**	**0.07**	0.58	0.56	0.63	0.58	0.66	0.59
PG1/PBG2(166bp)	0.25	0.29	**0.05**	**0.05**	0.61	0.57	0.57	0.60
PBG1/PG2(205bp)	0.32	0.037	0.58	0.59	**0.04**	**0.04**	0.49	0.51
PBG1/PBG2(262bp)	0.36	0.36	0.37	0.42	0.12	0.15	**0.03**	**0.04**

Various combinations of labeled and unlabeled amplicon with varied length were subjected to PCR-PHFA. Labeled amplicons were prepared from normal gene, and unlabeled amplicons were prepared from normal (N) or mutant (M) gene. Labeled and 20-fold excess amount of unlabeled amplicons were denatured at 98°C for 10 min. Then hybridization mixtures were cooled slowly (**A**: 98° to 70°C, 1°C/10 min) or quickly (**B**: 98° to 40°C, in 1 min). The resulted mixtures were processed for the enzymatic quantification and absorbance at 405 nm corrected by subtraction of the background are shown. The experiment was done with duplicate and average values are listed. The numbers with bold letter indicate the combinations of labeled and unlabeled amplicons with same length.

substrate. Quantitation is achieved using an unmodified microtiter plate reader.

As a model system, we detected a single base substitution, Glu(GAG) to Val(GTG), at codon 6 of human β-globin gene. The plasmid pBR322-HbS, carrying the mutated gene, and the normal human gene were used as templates and amplified with appropriate sets of primers (listed in Table 1). One microliter of a labeled amplicon (probe), prepared from normal gene, and 20 μl of unlabeled amplicon (sample) prepared from normal or mutant genes were mixed. After denaturation, the mixtures were cooled slowly (Table 2-A) or quickly (Table 2-B) as indicated in the legend. The regenerated double labeled dsDNA was detected enzymatically. As shown in Table 2-A, when labeled and unlabeled amplicons were the same in length, a single base substitution resulted in a drastic difference in A_{405}. In contrast, when the probe and the sample differed in length, there was no clear distinction between the normal and the mutant genes. When a probe sequence was included in a sample sequence and the difference in length between the two was relatively small, a single base substitution was still detectable, although not overwhelmingly convincing.

When the hybridization mixtures were cooled quickly, a single base substitution did not cause any significant difference in A_{405}

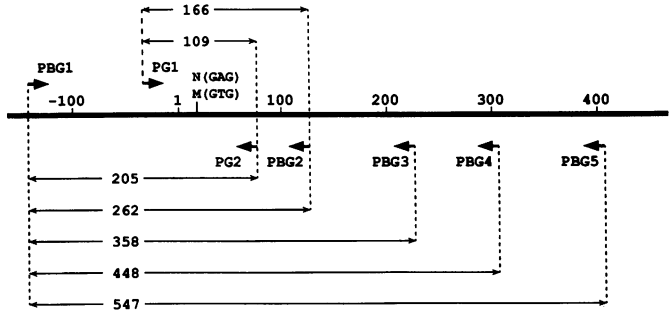

Figure 2. Arrows with bold line indicate the locations of primers. The numbers between the arrows are the number of base pairs generated with each set of primers. A single base substitution is located at the position corresponding to the 6th amino acid residue of the human β-globin gene. N, Normal gene (GAG, Glu); M, Mutant gene (GTG, Val).

(Table 2-B). Under this condition, the combination of labeled and unlabeled amplicons of the same length generated less double labeled dsDNA. When labeled and unlabeled amplicons had no mismatch but had difference in length, lower A_{405} due to strand

Table 3. Effect of length of PCR amplicon on the discrimination of single base substitution by PCR-PHFA

Labeled amplicon	Unlabeled amplicon		
	normal	mutant	mutant/normal
PBG1/PG2 (205bp)	0.055 ± 0.008	0.812 ± 0.029	14.8
PBG1/PBG2 (262bp)	0.054 ± 0.006	0.601 ± 0.088	11.1
PBG1/PBG3 (358bp)	0.055 ± 0.012	0.210 ± 0.016	4.2
PBG1/PBG4 (448bp)	0.050 ± 0.006	0.201 ± 0.016	4.0
PBG1/PBG5 (547bp)	0.038 ± 0.005	0.153 ± 0.010	3.8

Single base substitution in relatively long amplicon was detected by PCR-PHFA. Length of amplicon generated with each primer set are listed in parentheses. Labeled amplicons were prepared with biotin and DNP primers, and unlabeled amplicons were prepared with aminoalkylated primers. The procedures of hybridization and detection are identical to table 2-(A). The experiment was done with triplicate and means ± standard deviation are listed. The ratios between absorbance at 405 nm of normal and mutant are also listed.

Table 4. Typing of HLA-DPB1 alleles by PCR-PHFA

Labeled	Unlabeled sample (standard DNA)								
probe	02012	0202	0301	0401	0402	0501	0901	1301	1901
02012	**0.12**	0.71	0.98	0.85	0.32	0.96	1.04	1.03	0.84
	(0)	(3)	(14)	(3)	(1)	(7)	(12)	(14)	(6)
0202	0.94	**0.12**	1.25	0.94	0.94	0.94	1.12	1.12	1.05
	(3)	(0)	(17)	(5)	(4)	(5)	(13)	(10)	(7)
0301	0.91	0.80	**0.11**	0.90	0.80	0.74	0.61	0.71	0.75
	(14)	(17)	(0)	(16)	(13)	(12)	(3)	(8)	(11)
0401	0.93	0.90	1.05	**0.09**	0.77	0.89	1.01	0.96	0.91
	(3)	(5)	(16)	(0)	(3)	(8)	(16)	(12)	(9)
0402	0.47	0.85	0.98	0.69	**0.12**	0.95	1.00	1.02	0.94
	(1)	(4)	(13)	(3)	(0)	(7)	(13)	(15)	(8)
0501	0.88	0.78	0.89	0.84	0.84	**0.09**	0.92	0.89	0.79
	(7)	(5)	(12)	(8)	(7)	(0)	(12)	(11)	(3)
0901	0.94	1.00	0.63	0.93	0.90	0.87	**0.10**	0.83	0.90
	(12)	(13)	(3)	(16)	(13)	(12)	(0)	(7)	(9)
1301	0.86	0.84	0.77	0.86	0.92	0.94	0.83	**0.09**	0.82
	(14)	(10)	(8)	(12)	(15)	(11)	(7)	(0)	(9)
1901	0.62	0.64	0.72	0.65	0.64	0.56	0.69	0.66	**0.09**
	(16)	(7)	(11)	(9)	(8)	(3)	(9)	(9)	(0)

The numbers of each probe corresponding to the DPB1 allele type. The numbers in upper row are the absorbance at 405 nm. Those in parentheses indicate the number of base substitutions between the probe and sample. Combinations of the same probe and sample are indicated by bold letter.

Table 5. HLA-DPB1 DNA typing of blood samples by PCR-PHFA

Labeled probe	Unlabeled sample (from blood)				
	#11	#14	#47	#57	#65
02012	**0.22**	**0.16**	0.91	0.93	**0.29**
0202	0.82	0.79	0.89	0.91	0.70
0301	0.70	**0.12**	0.94	0.68	0.69
0401	0.78	0.77	0.80	0.83	0.69
0402	0.49	0.43	0.83	0.91	0.45
0501	0.87	0.82	**0.03**	**0.20**	0.80
0901	**0.17**	0.66	0.96	**0.14**	**0.22**
1301	0.84	0.90	0.95	0.83	0.77
1901	0.85	0.91	0.82	0.79	0.76
Results of	*02012	*02012	*0501	*0501	*02012
PCR-RFLP	*0901	*0301	*0501	*0901	*0901
Results of	*02012	*02012	*0501	*0501	*02012
PCR-PHFA	*0901	*0301		*0901	*0901

Human genomic DNA, prepared from five Japanese individuals, were used for classification of HLA-DPB1 allele type by PCR-PHFA. Each probe had different absorbance at 405 nm when quantified directly without mixing with sample becauseof the variation of PCR efficiency (see Table 4). To compensate for this, the absorbance at 405 nm obatained by hybridization with sample was divided by those values obtained with H_2O. The absorbance at 405 nm in the table has been compensated. HLA-DPB1 allele type judged by this method and those of PCR-RFLP are listed in the table.

Table 6. Detection of single base substitution in a heterozygote by PCR-PHFA

(A)			
Labeled probe	Unlabeled sample	A_{405}	Average
	normal (20 μl)	0.104	
		0.107	0.106
normal (1 μl)	normal + mutant (10 μl each)	0.160	
		0.161	0.161
	mutant (20 μl)	0.926	
		0.913	0.921
(B)			
Unlabeled probe	Labeled sample	A_{405}	Average
	normal (1 μl)	0.113	
		0.127	0.120
normal (20 μl)	normal + mutant (0.5 μl each)	0.432	
		0.395	0.414
	mutant (1 μl)	0.980	
		0.975	0.978

Two combinations of probe and sample were compared to detect a single base substitution in a heterozygote. (A), labeled probe and unlabeled sample; (B), unlabeled probe and labeled sample. The assay was done with duplicate.

exchange should have been expected, however, the observed A_{405} was relatively high. This indicates that strand displacement between longer and shorter fragment occurred very efficiently even in these conditions. This explains why denatured PCR products tended to have lower sensitivity than single strand PCR products when captured with oligonucleotides (16,17).

We further examined the effect of target lengths on distinguishing a single base substitution by PCR-PHFA. Various lengths of amplicons containing a single base substitution were prepared. The positions of the primers are shown in Figure 2. The combination of 547 base pair amplicons showed the smallest difference in a ratio of mutant to normal at A_{405} (Table 3) and very low A_{405}. However, since intra-assay variability of this system was very small, it was still possible to detect a single base substitution when the normal gene was served as a control.

In conclusion, 1) precisely controlled temperature gradient is necessary to detect single base substitutions. 2) labeled and an unlabeled amplicons are required to be the same in length. 3) it was revealed that the shorter fragment had the higher preference for forming homoduplexes over heteroduplexes.

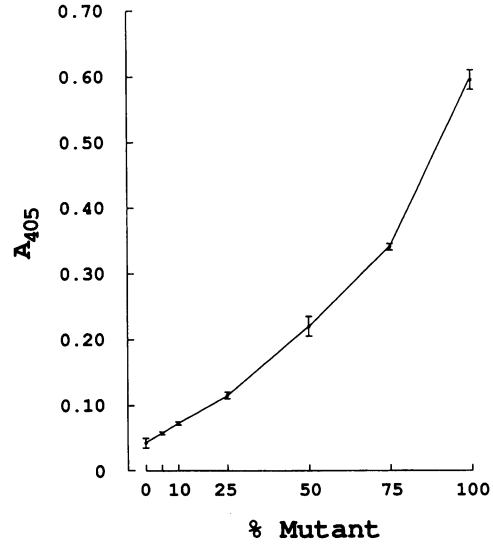

Figure 3. Detection of the activated *c*-H-*ras* gene in the presence of the normal gene. The unlabeled probe, prepared from normal gene, and the labeled samples consisting of mixtures of amplicons from normal and mutated genes, were analyzed by PCR-PHFA. The ratio of the unlabeled probe to the labeled sample was 20:1. The assay was done with triplicate. Each sample had slightly different absorbance at 405 nm when quantified directly without mixing with the probe because of the variation of PCR efficiency. To compensate for this, absorbance at 405 nm obtained by hybridization with the probe was divided by those values obtained with H_2O. The absorbance at 405 nm in the figure has been compensated.

HLA-DPB1 typing

Since the identity of labeled and unlabeled amplicons can easily be assessed by PCR-PHFA, this technique has been applied to HLA-DPB1 typing. The DPB1 gene has many nucleotide substitutions scattered in the second exon covering 280 base pairs and this makes it difficult to differentiate each type by conventional methods (18).

As authentic DNA, we cloned the second exon of each DPB1 type of genomic DNA into a plasmid vector by means of PCR with DPBAMP-A and DPBAMP-B (19) as primers (Table 1). At first, 9 types of the DPB1 gene were selected and tested whether or not each type could be identified. Double labeled and unlabeled amplicons were prepared by PCR using the primers listed in Table 1. Unlabeled (sample) and labeled (probe) amplicons were mixed with a ratio of 20:1, and analyzed by PCR-PHFA. As shown in Table 4, this system clearly differentiated each HLA-DPB1 allele. When the probe and the sample were of the identical HLA-DPB1 sequence, the value of A_{405} was low due to the dilution of double labeled with unlabeled molecules. On the other hand, the combinations of different alleles showed higher A_{405} values, indicating the preferential homoduplex formation under the temperature gradient. Certain combinations of different alleles showed mid-range levels of A_{405}. The numbers of base mismatched between the probe and sample are listed in parentheses. Less mismatches between probe and sample tended to result in lower A_{405}. Nevertheless, one base difference could be still easily detected by this method (compare 02012 vs 02012 as a complete match and 02012 vs 0402 as a single base mismatch). When probes and samples differed from each other by more than three base substitutions, the A_{405} value was equal to that obtained in the absence of sample (data not shown). This strongly suggests that the DNA molecules hardly form

heteroduplexes when two complementary strands differ by more than three base mismatches in the present system.

Considering the application to the typing of clinical samples, we prepared genomic DNA from human peripheral blood (15). One microgram of genomic DNA was PCR amplified with unlabeled primers, DPBAMP-A and DPBAMP-B(19). We analyzed five samples derived from five unrelated individuals and the results are listed in Table 5. These DNAs were also typed by PCR-RFLP method (20). One microliter of labeled amplicon (probe) and 15 μl of unlabeled amplicon generated from genomic DNA, were subjected to PCR-PHFA. Sample # 11 showed low A_{405} when hybridized with probes 02012 and 0901, indicating that this sample is a heterozygote with DPB1*02012 and *0901. Sample # 47 showed very low A_{405} only when hybridized with the 0501 probe. This result indicates sample # 47 has a DPB1*0501 homozygote or a heterozygote with DPB1*0501 and an allele type except these nine probes. According to the results of PCR-RFLP, this DNA is a DPB1*0501 homozygote. Although this sample showed lower absorbance than heterozygous sample (sample # 57) with the 0501 probe, it is not totally certain that this individual is a DPB1*0501 homozygote, as we discuss in the subsequent section. All five tested samples gave very clear results and completely coincided with those by PCR-RFLP. Sample # 11, which is heterozygous for DPB1*02012 and *0901, showed a medium A_{405} with the 0402 probe. As mentioned above, 02012 and 0402 have only one base difference in their sequences, and this gave a relatively low A_{405} due to heteroduplex formation. We observed a similar phenomenon in samples # 14 and # 65 with the 0402 probe.

Detection of a single base substitution in heterozygotes

Many inherited diseases, such as sickle cell anemia, are recessive traits. In order to diagnose this kind of diseases, heterozygous as well as homozygous mutants should be detected. As a model system, we used 205 bp human β-globin DNA fragments amplified with PBG1 and PG2 (Table 1 and Fig. 1). Ten microliter of 10-fold diluted labeled amplicon (probe) and 20 μl of unlabeled amplicon (served as a heterozygous sample) containing 10μl of the normal and 10μl of the mutant amplicons were analyzed by this method. As shown in Table 6-A, the distinction between the homozygous mutants and the others was clear. However, the difference between the heterozygote and the normal homozygotes were not obvious. If the efficiency of homoduplex formation is 100%, the labeled amplicon (probe) will be diluted with unlabeled homozygous normal sample at 1/21, and with the heterozygous mutant is at 1/11. Considering the background of this assay system's mainly due to the incomplete preference of homoduplex formation, this difference is not sufficient to judge a heterozygote.

In order to overcome this limitation, we tested the combination of a labeled sample and an excess of unlabeled probe. Twenty microliter of unlabeled amplicon (probe) prepared from normal gene, and 1μl of labeled amplicon (served as a heterozygous sample) containing 0.5 μl each of normal and mutant amplicons were subjected to PCR-PHFA. In this system, in a heterozygous sample, half of labeled molecule (mutant) will not be diluted, and the other half of the molecule (normal) will be diluted at 1/41. The observed A_{405} will be the sum of both values. As a result, the total amount of double labeled dsDNA will be about a half of the original amount. As shown in Table 6-B, the heterozygote and the homozygote for the normal allele are clearly discriminated. Similarly, the mutant homozygote and the heterozygote showed distinguishable A_{405} values. In this modification, three genotypes were completely differentiated. By using this modified system, HLA-DPB1 genotype can be concluded whether a homozygote or a heterozygote with an unknown allele type.

Detection of an activated oncogene DNA in the presence of excess of normal DNA

To diagnose a cancer in a tissue biopsy, a mutated oncogene must be detected in the presence of normal DNA. As a model system, the detection of the activated *c*-H-*ras* gene in the mixture of normal and mutated *ras* gene was carried out. For probe DNA, a 20 fold excess of unlabeled amplicon was derived from the normal gene, pSK-2(13). Mixtures containing various ratios of activated, prepared from pKY-1(14),and normal DNA were analyzed as labeled samples. As shown in Fig. 3, this method detected the activated *ras* gene in the sample consisting of at least 10% mutated DNA when the normal gene was served as a control. Furthermore, since the percentage of mutated DNA in the sample and A_{405} showed a good correlation, the content of mutated DNA in the sample can be estimated roughly by using this as a calibration curve.

PCR-PHFA for clinical diagnosis

In this paper, we describe a simple and reliable method for detecting single base substitutions at any positions in PCR amplicons. In order to detect the identical sequence to probe DNA in a sample, such as in HLA-DPB1 typing, the combination of labeled probe and unlabeled sample can be used. On the other hand, to detect diverse sequences in a sample, such as the case for activated oncogene, the combination of unlabeled probe and labeled sample can be used.

In terms of sensitivity, PCR with SSP can detect one percent of mutant cells in normal population (21). However, this technique can be only applied to already known mutations. For the detection for unspecified mutations, many PCR reactions with different sets of primers are required.

Although some techniques, such as PCR-SSCP and PCR-DGGE can identify unknown mutations in a PCR amplicon, they require optimization of conditions, such as temperature during electrophoresis, gel composition etc., for fine resolution, and the conditions differ for each target DNA. Furthermore, a DNA fragment with a different sequence does not always migrate at a different position. Thus although the diversity of two DNAs can be concluded by these techniques, the identity of two DNAs can not be confirmed. The reasons mentioned above may limit these techniques for application to routine DNA diagnosis for a large number of samples.

Since the system described here, PCR-PHFA, requires no expensive apparatus and gives clear results, it will be a powerful technique for routine DNA diagnosis in clinical laboratories and for use in many fields in molecular genetics.

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