Available online at www.sciencedirect.com
ScienceDirect
Analytica Chimica Acta 597 (2007) 97–102
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Fluorescence detection of single-nucleotide polymorphisms with two simple and low cost methods: A double-DNA-probe method and a bulge form method

Na Li [a], Ling Mei [a], Yu Xiang [a], Aijun Tong [a,*], Seiichi Nishizawa [b], Norio Teramae [b]

[a] *The Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Department of Chemistry, Tsinghua University, Beijing 100084, China*
[b] *Department of Chemistry, Graduate School of Science, Tohoku University, Aoba-ku, Sendai 980-8578, Japan*

Received 12 January 2007; received in revised form 8 June 2007; accepted 14 June 2007
Available online 19 June 2007

Abstract

Two 10-mer DNA probes, or one 20-mer DNA probe, respectively, hybridize with a 21-mer target DNA to form a vacancy or bulge opposite the target nucleotide. The former double-DNA-probe method and the latter bulge form method are applicable to the detection of single-nucleotide polymorphisms (SNPs). A small fluorescent dye enters into the vacancy or bulge and binds with a target nucleotide via a hydrogen bonding interaction, which causes fluorescence quenching. The interaction between fluorescent dye and the target nucleotide is confirmed by measuring the melting temperature and fluorescence spectra. The fluorescent dye, ADMND (2-amino-5,7-dimethyl-1,8-naphthyridine), is found to selectively bind with C over A or G. The methods proposed here are economic, convenient, and effective for the fluorescence detection of SNPs. Finally, the double-DNA-probe method and bulge form method are successfully applied to the detection of C/G and C/A mutations in the estrogen receptor 2 gene and progesterone receptor gene using ADMND.

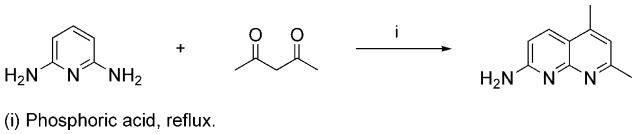
Keywords: Single-nucleotide polymorphisms; Fluorescence detection; Double-DNA-probe method; Bulge form method

1. Introduction

Single-nucleotide polymorphisms (SNPs) are the most common type of human genetic mutation, which account for 90% of sequence variants [1]. High density and mutational stability of SNPs make them particularly useful DNA markers for population genetics and for mapping susceptibility genes of complex diseases [2,3]. Such studies will involve analysis of large numbers of SNPs in hundreds or thousands of individuals [4,5], and thus high-throughput SNP typing method is required. It is anticipated that SNP analysis will be used to diagnose common diseases, to determine disease susceptibility, and to predict an individual's response to therapeutic treatment. To meet these anticipations, quick, simple, and low cost methods for SNP typing are essential. The current SNP typing methods can be roughly classified into two categories: enzyme-based methods and hybridization-based methods [6,7]. Since the former methods usually require complicated experimental procedures, hybridization-based methods are more attractive because of the simplicity in their experimental procedures and several rapid SNP typing methods have been developed such as TaqMan [8], Molecular Beacon [9], and iFRET [10]. In these SNP detection techniques, fluorescent dyes are covalently bound to an oligo-DNA strand. Such hybridization-based methods require labeling of DNA strands or ddNTP by fluorescent dyes [11–13], and the fluorophore-labeled DNAs are expensive. Recently, SNP typing methods without labeling DNAs by fluorophores have been reported using small molecules which bind to the target nucleotide selectively. For example, Nakatani et al. [14,15] have reported on the detection method for a single guanine bulge and guanine–guanine mismatches using 1,8-naphthylidine derivatives which can bind with guanine through hydrogen bond. Binding of fluorescent dye to target nucleotides was confirmed by absorption spectrometry, circular dichroism (CD) and melting temperature measurements. Teramae's group has proposed to use an oligo-DNA duplex containing an

* Corresponding author. Tel.: +86 10 62787682; fax: +86 10 62787682.
E-mail address: tongaj@mail.tsinghua.edu.cn (A. J. Tong).

0003-2670/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2007.06.031



(i) Phosphoric acid, reflux.

Scheme 1. Synthesis of ADMND.

apurinic or apyrimidinic site (AP site), as a recognition field for a target nucleotide [16–18]. Fluorescent ligands can bind to the target nucleotide opposite an AP site, thus realizing fluorescence detection without labeling fluorophores into the oligo-DNAs. Although their approach is quite effective and convenient, the synthesis of AP site-containing DNA strands is tedious and commercially ordered products are expensive. Therefore, finding a more convenient and economical method for fluorescence detection of SNPs still challenges researchers.

This paper reports two simple, effective, and inexpensive methods for detecting SNPs, *i.e.*, a double-DNA-probe method and a bulge form method. In these methods, the target DNA strand contains one extra nucleotide compared to the probe DNA strand(s) and thus a vacancy or bulge is formed when the target DNA hybridizes with the DNA probe(s). A fluorescent dye can enter into the vacancy or bulge and can bind selectively to the target nucleotide by forming hydrogen bonds. Thus, labeling DNA or AP site-containing DNA strands or DNA immobilization are not fully required. In the present study, 2-amino-5,7-dimethyl-1,8-naphthyridine (ADMND) [19,20] was used as fluorescent dye and its binding behavior with a target nucleotide in DNA duplexes was examined by UV–vis absorption and fluorescence spectroscopies.

2. Experimental

2.1. Materials

ADMND (2-amino-5,7-dimethyl-1,8-naphthyridine) was synthesized in one step by reacting 2,6-diaminopyridine with 2,4-pentanedione, according to the literature [19,20]. The reaction scheme is shown in Scheme 1.

All oligo-DNAs used were purchased from Takara Corporation (Dalian, China). Concentration of each oligo-DNA was determined according to the literature [21] using the molar extinction coefficient calculated from absorbance at 260 nm. The other reagents were commercially available analytical grade and were used without further purification.

Aqueous solutions of fluorescent dyes were prepared as follows: 8.6 mg of ADMND was dissolved in 1 mL of hot water and diluted to give a 1.0 mmol L^{-1} solution.

2.2. Measurements

Fluorescence spectra were measured on a JASCO FP-6500 spectrofluorophotometer equipped with an ETC-273T peltier thermostatted single cell holder whose temperature was set to 5 °C. Both excitation and emission slits were set at 3 nm. Absorption spectra were recorded on a JASCO V-550 UV-VIS spectrophotometer equipped with an ETC-505 peltier thermostatted single cell holder. All spectra were measured using a microcell whose optical path length is 1 mm. pH values were measured on a Model pHS-3C pH meter from Leici Corporation (Shanghai, China). Doubly distilled water was used throughout the experiments. All measurements were performed in 10 mM sodium cacodylate buffer solutions (pH 7.0) containing 100 mmol L^{-1} NaCl and 1.0 mmol L^{-1} EDTA.

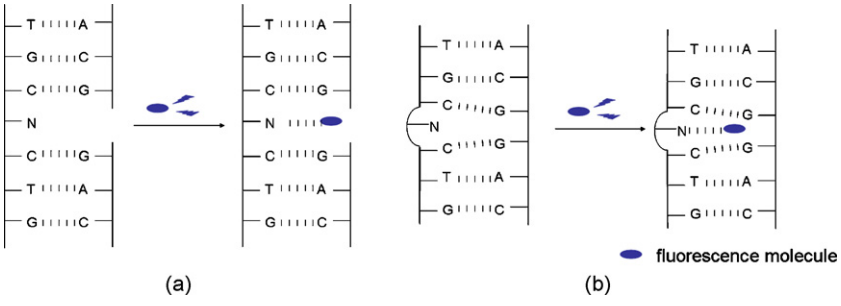
2.3. Methods

2.3.1. Double-probe method

As illustrated in Scheme 2(a), the two probes are simple DNA single strands containing 10 nucleotides. Their nucleotides sequences are designed so as to be a complimentary match with a target DNA strand except for the target nucleotide, which can form a vacant site opposite the target nucleotide N (N denotes T, C, A, or G). Their sequences are given as follows: probe 1, 5′-GCAACGATAG-3′; probe 2, 5′-GTACATCCAG-3′; target, 5′-CTATCGTTGCNCTGGATGTAC-3′. After hybridization of two probe strands with the target DNA strand, double strands are formed having a vacant site opposite the target nucleotide. A small fluorescent dye then enters into the vacant site and can bind with the nucleotide via hydrogen bonds, which induces the change in fluorescence of the fluorescent dye.

2.3.2. Bulge form method

Bulges are a well known structural element in RNA or DNA and can be either a missing base, or an extra one that has been inserted during the DNA copying process. In a bulge form method, a probe DNA strand (5′-GTACATCCAGGCAACGATAG-3′) hybridizes with a target DNA strand (5′-CTATCGTTGCNCTGGATGTAC-3′, N = target nucleotide (T, C, A, G)) and a bulge is formed opposite the target nucleotide.



Scheme 2. Schematic illustration of double-probe (a) and bulge form (b) methods. N is the target nucleotide.

Table 1
Melting temperatures of DNA duplexes in the absence ($T_m(-)$) and presence ($T_m(+)$) of ADMND by double-probe method

	$T_m(-)$ (°C)	$T_m(+)$ (°C)	ΔT_m (°C)
C	45.7	49.1	3.4
A	46.8	47.6	0.8
G	46.5	47.2	0.7
T	45.2	47.4	2.2
Fully matched DNA duplex	70.8	71.1	0.3

C, A, T and G are target nucleotides and the DNA sequences are indicated in Section 2. [DNA duplex], 3.0×10^{-5} M; [ADMND], 5.0×10^{-4} M.

A fluorescent dye thus can enter into the oligo-DNA duplex and can form hydrogen bonds with the target nucleotide, which induces the change of fluorescence as shown in Scheme 2(b).

For comparison with the double-probe method and bulge form method, a fully matched DNA duplex (5′-CTATCGTTGCCCTGGATGTAC-3′ and 5′-GTACATCCAGGGCAACGATAG-3′) and a single-strand DNA (5′-GTACATCCAGGGCAACGATAG-3′) are also examined in the experiments.

3. Results and discussion

3.1. Melting temperature (T_m) measurements of oligo-DNA duplexes with and without fluorescent dyes

The thermal stabilities of oligo-DNA duplexes in the presence and absence of ADMND are shown in Tables 1 and 2; they were assessed by measuring the melting temperature (T_m). In the case of the double-probe method (Table 1), a significant increase in T_m is observed when the target nucleotide is C or T. The increase in T_m (ΔT_m) reaches 3.4 °C for the target nucleotide C, and 2.2 °C for T, whereas ΔT_m for A and G as well as for a fully matched DNA duplex shows only a small increase. This result indicates that ADMND can interact with a target nucleotide strongly when the nucleotide is C or T. In the case of the bulge form method (Table 2), the increase in T_m is not so significant but a larger increase in ΔT_m is recognized for the nucleotides C or T than for A or G, similar to the case of the double-probe method except for reversed order for C and T. This result indicates that ADMND can interact more with the target nucleotides C or T than with A or G at the bulge.

Table 2
Melting temperatures of DNA duplexes in the absence ($T_m(-)$) and presence ($T_m(+)$) of ADMND by bulge form method

	T_m (-) (℃)	T_m (+) (℃)	ΔT_m (℃)
		63.8	
C	62.7		1.1
A	62.4	62.3	-0.1
G	62.4	62.7	0.3
T	60.3	61.9	1.6

C, A, T and G are target nucleotides and the DNA sequences are indicated in Section 2. [DNA duplex], 3.0×10^{-5} M; [ADMND], 5.0×10^{-4} M.

3.2. Fluorescence detection of SNPs by the double-probe and bulge form methods

Applicability of the double-probe method for SNP was examined by using ADMND as a fluorescent dye. Fig. 1(a) shows the fluorescence spectra of ADMND (5 μM) in 10 mM cacodylate buffer solution (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. ADMND exhibits a strong emission band around 400 nm (spectrum 1), but its fluorescence is quenched remarkably upon addition of 5 μM DNA duplexes when the nucleotide opposite the vacant site is C (spectrum 6). The degree of fluorescence quenching greatly depends on the target nucleotide opposite the vacant site. When the target nucleotide is C, the fluorescence is most effectively quenched (ca. 74%), while the fluorescence quenching is moderate (ca. 20%) when the nucleotide is G (spectrum 4) or A (spectrum 5). Fluorescence quenching of ca. 20% is also recognized upon addition of a single-strand DNA (spectrum 2) and a fully matched DNA duplex (spectrum 3). This result may be ascribed to the intercalation of ADMND into DNA strands and/or the interaction between the phosphodiester group of a DNA strand and ADMND. In the case of the bulge form method, similar results are obtained as for the double-probe method as shown in Fig. 1(b). Although fluorescence quenching of ADMND is not so remarkable in the bulge form method compared with the results shown in Fig. 1(a), it is possible to easily distinguish the target nucleotide C from A or G. Since 2-amino-1,8-naphthyridine derivatives were reported to bind selectively with C over other nucleotides at the abasic site in a DNA duplex [16], the C-selective quenching of ADMND in Fig. 1(a) and (b) can be ascribed to the formation of hydrogen bonds between ADMND and C. In addition, π-stacking interaction between ADMND and the adjacent nucleotides can enhance the binding ability of ADMND with a DNA strand containing a target nucleotide. The nucleotide T can also cause fluorescence quenching of ADMND to some extent, being 64% by the double-probe method and 32% by the bulge form method. Thus, ADMND is not suitable for the detection of C/T mutation. However, ADMND is applicable in the detection of C/A or C/G mutation by the double-probe and bulge form methods, especially from the viewpoint of greatly reduced experimental cost compared with an AP site-containing DNA probe method [16].

Binding behavior of ADMND with C was examined quantitatively by fluorescence titration experiments. Fig. 2 shows the dependence of ADMND fluorescence intensity at 400 nm on DNA concentration by using both the double-probe (A) and the bulge form (B) methods, where F and F_0 denote the fluores-

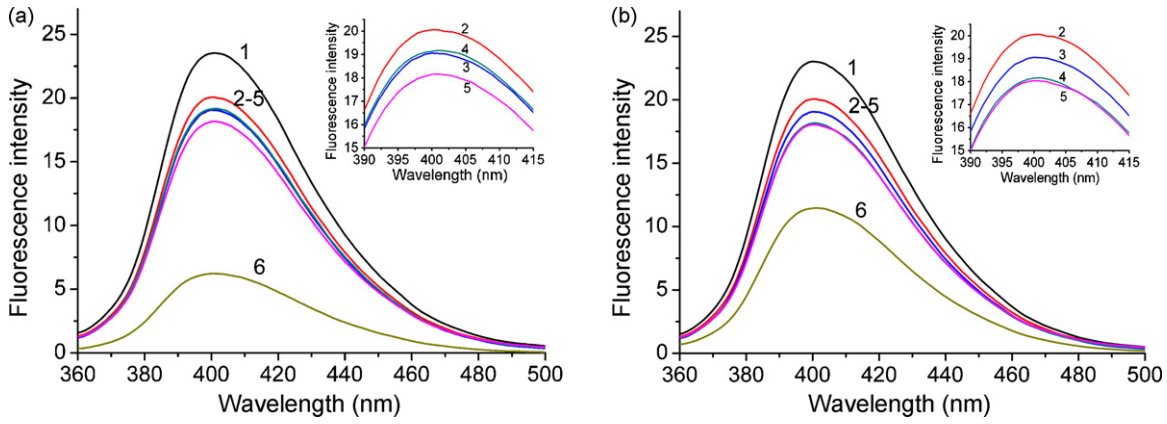


Fig. 1. Fluorescence spectra of ADMND (5.0×10^{-6} M) in the presence of 5.0×10^{-6} M oligo-DNA. (a) Double-probe method; (b) bulge form method. 1, DNA free; 2, single-strand DNA; 3, fully matched DNA duplex; 4–6 are in the presence of target DNA with a nucleotide N with 4, N = G; 5, N = A; 6, N = C. The DNA sequences are indicated in Section 2. Sample solutions were buffered to pH 7.0 with 10 mM sodium cacodylate containing 100 mM NaCl and 1.0 mM EDTA. Excitation wavelength was 347 nm.

cence intensity of ADMND in the presence and absence of a DNA duplex, respectively. In both methods, concentration of ADMND was 10 μM. In the case of the double-probe method (curve A), the fluorescence is almost completely quenched upon addition of 20 μM DNA duplex and an inflection point can be recognized when the concentration of DNA duplex is 10 μM, suggesting 1:1 complexation between ADMND and DNA duplex. In the case of the bulge form method (curve B), the change in fluorescence intensity upon addition of DNA duplex is gradual compared with the double-probe method because of the weaker binding affinity of ADMND with DNA duplex containing a bulge than DNA duplex containing a vacant site in the double-probe method. Titration end point is also recognized when the concentration of DNA duplex is ca. 10 μM. The 1:1 binding constants between ADMND and an oligo-DNA duplex, calculated from non-linear regression of the titration curve, are $5.2 \times 10^6\ M^{-1}$ in the double-probe method and $3.4 \times 10^5\ M^{-1}$ in the bulge form method. Curve C is obtained by titration of ADMND with a fully matched DNA duplex. It is seen that fluorescence of ADMND is also partially quenched upon addition of a fully matched DNA duplex due to its intercalating feature. However, the degree of quenching is much lower than the DNA duplex containing a vacant site or bulge. For example, when the concentration of a DNA duplex is 10 μM, F/F_0 is only about 0.83 for a fully matched DNA duplex, while F/F_0 is 0.06 in the double-probe method and 0.40 in the bulge form method, when the target nucleotide is C. Accordingly, both the double-probe and bulge form methods might be applicable to the detection of C-related SNPs by considering the concentration range of the DNA duplex.

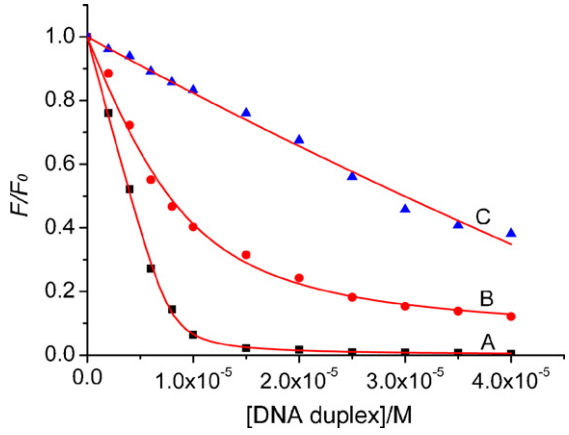


Fig. 2. Non-linear regressive analyses of the changes in fluorescence intensity ratios at 400 nm. (A) double-probe method (target nucleotide: C); (B) bulge form method (target nucleotide: C); (C) fully matched DNA duplex. F and F_0 denote the fluorescence intensities of ADMND in the presence and absence of DNA duplex. The ADMND concentration was 1.0×10^{-5} M, other conditions were the same as in Fig. 1.

3.3. Effect of flanking bases

It is well known that displacement of base pairs can be caused depending on the sequence [22], which means that the sequence can affect the π-stacking interaction between a fluorescent dye bound to a target base and flanking bases. Accordingly, fluorescence of ADMND at the vacant site or bulge in a DNA duplex may be affected by the neighboring bases, although complementary hydrogen bonds are formed between ADMND and a target nucleobase. To examine the effect of flanking bases on the fluorescence quenching of ADMND, base selectivity of ADMND was examined using another DNA strand with A and T as neighboring bases, 5′-CTATCGTTGTNATGGATGTAC-3′ (N = target nucleotide T, C, A, or G) in the double-probe and bulge form methods. As shown in Fig. 3, almost the same quenching degree is observed compared with the results shown in Fig. 1, indicating the effect of flanking bases is not significant and hydrogen bonding plays a crucial role in the present methods.

3.4. Analysis of cytosine related mutation present in genes

The double-probe method and bugle form method were applied to the detection of cytosine related mutation which is present in the estrogen receptor 2 gene and progesterone receptor

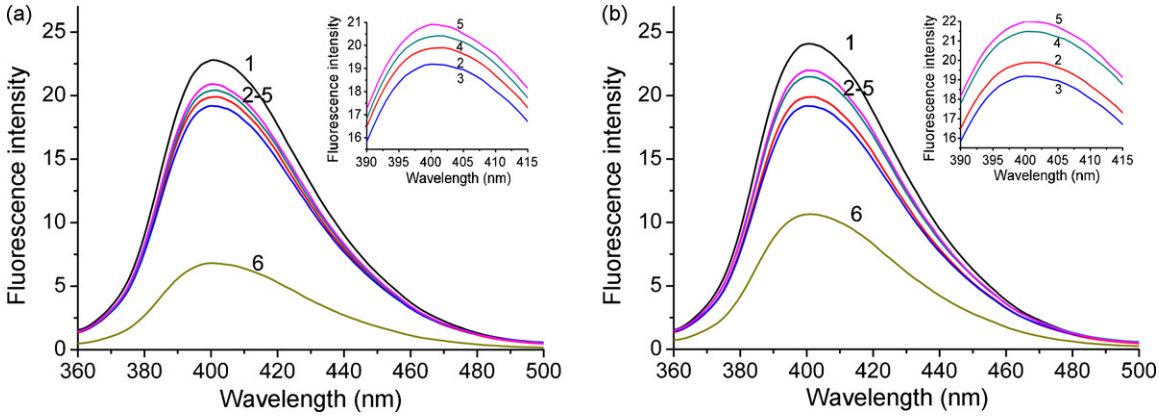


Fig. 3. Fluorescence spectra of ADMND (5.0×10^{-6} M) in the presence of 5.0×10^{-6} M oligo-DNA with A and T as flanking bases (target DNA: 5′-CTATCGTTGTNATGGATGTAC-3′). (a) Double-probe method (probe 1, 5′-ACAACGATAG-3′; probe 2, 5′-GTACATCCAT-3′); (b) bulge form method (probe, 5′-GTACATCCATACAACGATAG-3′). 1, DNA free; 2, single-strand DNA; 3, fully matched DNA duplex; 4–6 are in the presence of target DNA with a nucleotide N with 4, N = G; 5, N = A; 6, N = C. Other conditions were the same as in Fig. 1.

Table 3
Fluorescence quenching efficiency ($(F_0 - F)/F_0$, %) for two SNPs by the double-probe method (method A) and bugle form method (method B)

SNPs	DNA sequence	$(F_0 - F)/F_0$ (%)	
		Method A	Method B
rs1255998			
Probe 1	5′-GACGGTTGTC-3′		
Probe 2	5′-AGTCGACACG-3′		
Probe 3	5′-AGTCGACACGGACGGTTGTC-3′		
Target	5′-GACAACCGTC[C/G]CGTGTCGACT-3′	CG-c: 76; CG-g: 21	CG-c: 66; CG-g: 36
rs2020876			
Probe 1	5′-CGACATGCTG-3′		
Probe 2	5′-ACTTTCTAAG-3′		
Probe 3	5′-ACTTTCTAAGCGACATGCTG-3′		
Target	5′-CAGCATGTCG[C/A]CTTAGAAAGT-3′	CA-c: 71; CA-a: 10	CA-c: 52; CA-a: 28

Probes 1 and 2 were used in the double-probe method and probe 3 was used in the bulge form method. CG-c: C/G mutation with target nucleotide is cytosine; CG-g: C/G mutation with target nucleotide is guanine; CA-c: C/A mutation with target nucleotide is cytosine; CA-a: C/A mutation with target nucleotide is adenine. Concentrations of ADMND and DNA duplex were both 5.0×10^{-6} M. Other conditions were the same as in Fig. 1.

genes. It has been suggested that the genetics of breast cancer is related to the estrogen and progesterone receptors [23]. The sequences of two SNPs, rs1255998 (C/G mutation) and rs2020876 (C/A mutation), examined here are listed in Table 3. Fluorescence response of ADMND when applied to the detection of C/G and C/A mutation is also summarized in Table 3. It is seen that ADMND can recognize the transformation between C and G, as well as C and A by both methods although the bulge form method gives moderate results.

4. Conclusion

In conclusion, the double-probe and bulge form methods were examined for their ability to detect single-nucleotide polymorphisms (SNPs) by using a small fluorescent dye ADMND. The methods require no labeling of DNA strands and thus economic and cost-effective SNPs detection can be realized. The double-probe method was found to be superior to the bulge form method. Using the double-probe method and bulge form method, ADMND could be successfully applied to the detection of C/G and C/A mutations in the estrogen receptor 2 gene and the progesterone receptor genes. By choosing a proper fluorescent dye with high selectivity for a target nucleotide and by combination with PCR amplification, the detection limit may be further improved and other kinds of SNPs (not only C/A or C/G mutations) may be conveniently and economically detected by the double-probe method proposed here.

Acknowledgement

The authors thank the National Natural Science Foundation of China for financial support (no. 20375021).

References

[1] F.S. Collins, L.D. Brooks, A. Chakravarti, Genome Res. 8 (1998) 1229.
[2] A.J. Brookes, Gene 234 (1999) 177.
[3] S. Ye, S. Dhillon, X.Y. Ke, A.R. Collins, I.N.M. Day, Nucleic Acids Res. 29 (2001) e88.
[4] D. Cyranoski, Nature 410 (2001) 1013.
[5] M.C. Lu, J.G. Hall, M.R. Shortreed, L. Wang, W.T. Berggren, P.W. Stevens, D.M. Kelso, V. Lyamichev, B. Nen, J.L. Skinner, L.M. Smith, J. Am. Chem. Soc. 124 (2002) 7924.

[6] B.W. Kirk, M. Feinsod, R. Favis, R.M. Kliman, F. Barany, Nucleic Acids Res. 30 (2002) 3295.
[7] A.C. Syvanen, Nat. Rev. Genet. 2 (2001) 930.
[8] K.J. Livak, J. Marmaro, J.A. Todd, Nat. Genet. 9 (1995) 341.
[9] S. Tyagi, D.P. Bratu, F.R. Kramer, Nat. Biotechnol. 16 (1998) 49.
[10] W.M. Howell, M. Jobs, A.J. Brookes, Genome Res. 12 (2002) 1401.
[11] T. Maruyama, T. Shinohara, H. Ichinose, M. Kitaoka, N. Okamura, N. Kamiya, M. Goto, Biotechnol. Lett. 27 (2005) 1349.
[12] C.R. Twist, M.K. Winson, J.J. Rowland, D.B. Kell, Anal. Biochem. 327 (2004) 35.
[13] K. Takatsu, T. Yokomaku, S. Kurata, T. Kanagawa, Nucleic Acids Res. 32 (2004) e60.
[14] K. Nakatani, S. Sando, I. Saito, J. Am. Chem. Soc. 122 (2000) 2172.
[15] K. Nakatani, S. Sando, H. Kumasawa, J. Kikuchi, I. Saito, J. Am. Chem. Soc. 123 (2001) 12650.
[16] K. Yoshimoto, S. Nishizawa, M. Minagawa, N. Teramae, J. Am. Chem. Soc. 125 (2003) 8982.
[17] K. Yoshimoto, C.Y. Xu, S. Nishizawa, T. Haga, H. Satake, N. Teramae, Chem. Commun. (2003) 2960.
[18] S. Nishizawa, N.B. Sankaran, T. Seino, Y.Y. Cui, Q. Dai, C.Y. Xu, K. Yoshimoto, N. Teramae, Anal. Chim. Acta 556 (2006) 133.
[19] J. Bernstein, B. Stearns, E. Shaw, W.A. Lott, J. Am. Chem. Soc. 69 (1947) 1151.
[20] Y. Xiang, A.J. Tong, Chem. Lett. 34 (2005) 926.
[21] J.D. Puglisi, I. Tinoco Jr., Method Enzymol. 180 (1989) 304.
[22] W.K. Olson, A.A. Gorin, X.J. Lu, L.M. Hock, V.B. Zhurkin, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 11163.
[23] B. Gold, F. Kalush, J. Bergeron, K. Scott, N. Mitra, K. Wilson, N. Ellis, H. Huang, M. Chen, R. Lippert, B.V. Halldorsson, B. Woodworth, T. White, A.G. Clark, F.F. Parl, S. Broder, M. Dean, K. Offit, Cancer Res. 64 (2004) 8891.