Bioorganic & Medicinal Chemistry 16 (2008) 7013–7020

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Acridone-tagged DNA as a new probe for DNA detection by fluorescence resonance energy transfer and for mismatch DNA recognition

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ARTICLE INFO

Article history:
Received 28 February 2008
Revised 14 May 2008
Accepted 15 May 2008
Available online 29 May 2008

Keywords:
Labeled DNA
Acridone
Fluorescence
Resonance energy transfer
DNA detection
Mismatch recognition

ABSTRACT

Acridone is highly fluorescent and stable against photodegradation, oxidation, and heat. It is also a small molecule with no charge, making it a promising fluorescent agent for use in a DNA probe. Thus, we have prepared 5′-terminal acridone-labeled DNAs by post-modification, and have examined their photophysical properties and their use as donors for a fluorescence resonance energy transfer (FRET) system in combination with a 3′-terminal dabcyl-tagged DNA as an acceptor, which can detect the target DNA by emission-quenching caused by FRET. The FRET with an acridone and dabcyl pair has been found to complement that with fluorescence and dabcyl and other fluorescence–quencher pairs. Significant amounts of quenching of the acridone emissions by guanine in the DNA were observed when guanine was close to acridone, which can be applied as a quencher-free probe for the detection of special sequence of DNA. The DNA bearing acridone at the C5 position of inner thymidine could discriminate the opposite T-T base mismatch, although enhancement of discrimination ability is needed for the practical use of SNP typing.

1. Introduction

DNA tagged with a fluorescent agent is widely used as DNA probes for the detection of special DNA, studies of protein interactions, and single-nucleotide polymorphism (SNP) typing. Various methods for the synthesis of fluorescence-labeled DNA have been reported in the past two decades.[1–7] Fluorescein, cyanine dyes, and pyrene derivatives are widely used as labeling agents for DNA and proteins. However, these molecules have disadvantages such as easy photodegradation under irradiation upon excitation of the fluorophore and fluorescence quenching under acidic or basic conditions. These conventional fluorescent agents are rather bulky molecules with negative or positive charges that may interfere with their biological activity.

Acridone is highly fluorescent and stable against photodegradation, oxidation, and heat.[8,9] Moreover, it is a small molecule with no charge. Several acridone derivatives have been used as fluorescent labels for peptides,[10] amino acids,[11] antibodies,[12] and substrates for catalysis.[13] We have recently shown that acridone-tagged thymidine nucleotide can be incorporated into DNA during the polymerase chain reaction, enzymatically forming multiacridone-labeled DNA.[14] Saito and coworkers have reported that acridone-tagged DNA can be used as a base-discriminating fluorescent probe for single-nucleotide polymorphism, although it has some sequence limitations.[15] The acridone derivative has a high quantum yield of fluorescence at 420–480 nm by excitation at 360–400 nm.[8,9] These wavelengths of the emissions indicate that acridone could be useful as a donor in a fluorescent energy transfer (FRET) system in combination with dabcyl (4-[4-(dimethylamino)phenylazo]benzoyl) as an acceptor molecule, which has absorption at 420–520 nm. However, no experimental study on the FRET system using an acridone and dabcyl pair has been reported, although possibility of FRET using an acridone-labeled amino acid was proposed by Szymanska et al.[11] The FRET system using several pairs of donors and quenchers has been explored,[16,17] and fluorescein as a donor and dabcyl as an acceptor have been applied to the study of DNA and proteins.[18–21] Thus, we undertook preparation of acridone-labeled DNA by post-modification and examined its use as a donor for the FRET system in combination with a dabcyl-tagged DNA as an acceptor, in which a special target DNA can be detected by FRET. The FRET with an acridone and dabcyl pair has been found to be somewhat superior and complements that with a pair of fluorescence and dabcyl and other fluorescence–quencher pairs, although significant quenching of the acridone fluorescence by guanine in the DNA was observed depending on the DNA sequence. We report herein the synthesis of acridone-tagged DNAs, their photophysical properties, and a FRET system comprising an acridone and dabcyl pair, and compare it with a fluorescein–dabcyl system. We also evaluated acridone-tagged DNA as a fluorescent probe for mismatch DNA detection.

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doi:10.1016/j.bmc.2008.05.050

2. Results and discussion

2.1. Synthesis of acridone acetic acid and acridone-tagged DNA

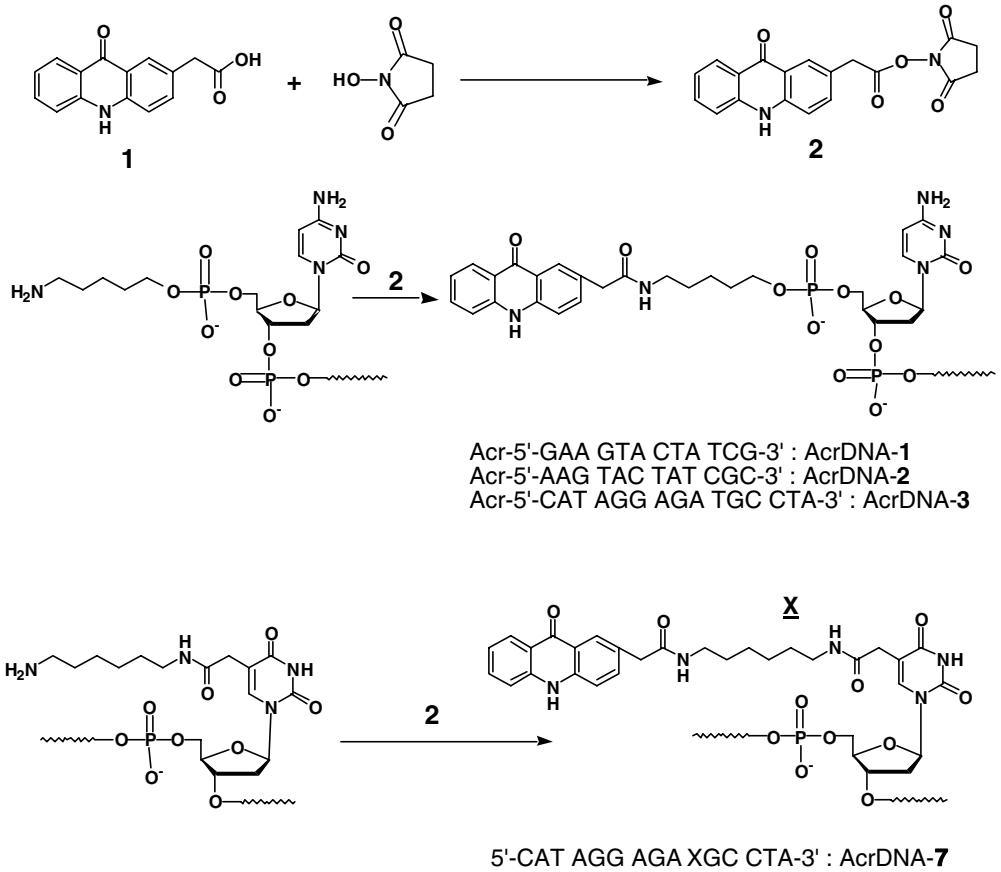
We prepared 2-acridone acetic acid (**1**) as an acridone-labeling agent.[14,22] Compound **1** was easily prepared from *o*-bromo benzoic acid and methyl *p*-aminophenyl acetate by Ullmann–Goldberg condensation, followed by cyclodehydration with polyphosphoric acid, and hydrolysis of the resulting methyl ester. Compound **1** was condensed with *N*-hydroxysuccinimide using DCC as a condensing agent forming activated *N*-hydroxysuccinimide ester (**2**), which was isolated as a yellow powder. The activated ester **2** can be kept in a desiccator stored in a freezer for long time, and can be used for the synthesis of acridone-tagged DNA without further purification. Alternatively, **2** was prepared just before the reaction with a terminal amino group of modified DNA and used in situ without isolation. The compound **2** was reacted with a modified DNA bearing an amino-linker at 5′-terminal position or at C5 position of thymidine residue by post-modification to introduce acridone into the DNA, as shown in Scheme 1. The acridone-tagged DNAs were characterized by MALDI-TOF mass spectrometry. The yields of the purified acridone-tagged DNAs were 13–52%, although we did not optimize the condensation and purification processes.

2.2. Photophysical properties of acridone acetic acid

We first examined photophysical properties of acridone acetic acid **1**. Figure 1A shows absorption of **1** in water, methanol, acetonitrile, and tetrahydrofuran. **1** has an absorption maximum at 388 and 407 nm in water. The absorption maximum of **1** shifts to a shorter wavelength in methanol. The extent of the shift is larger in a hydrophobic aprotic solvent like THF and acetonitrile. The fluorescence spectra of **1** in several solvents are shown in Figure 1B. Compound **1** showed strong fluorescence, peaks at 422 and 448 nm. The quantum yield of the fluorescence of **1** was 0.89 in water. However, the fluorescence intensity decreases significantly in an organic solvent, in the order of water > methanol > acetonitrile > THF. The decrease in fluorescence intensity is likely proportional to the hydrophobic character of the solvent. The fluorescence property of **1** in the organic solvent is in accordance with that of other acridone derivatives.[8,9,11] The fluorescence property depending on the solvent indicates that acridone can be applied as a probe for the investigation of hydrophobicity of the surrounding microenvironment.

2.3. Photophysical properties of acridone-tagged DNA at 5′-terminal

Figure 2 illustrates fluorescence spectra of 5′-terminal acridone-tagged DNAs, AcrDNA-1, AcrDNA-2, and AcrDNA-3, at the same concentration of 0.25 μM in response to excitation at 388 nm. The fluorescence spectra were almost the same, but the fluorescence intensities varied greatly depending on differences in the DNA. AcrDNA-2, which has a 5′-terminal adenine close to the acridone moiety, showed strong fluorescence intensity. In contrast, AcrDNA-1, which bears a 5′-terminal guanine that is a neighbor of acridone, showed weak fluorescence intensity. AcrDNA-3 containing a 5′-terminal cytosine showed intermediate fluorescence intensity. The second neighboring bases of these three DNA were the same adenine. These results demonstrate that sequence of the DNA, especially nucleic acid bases adjacent to acridone, has a pro-



Scheme 1. Synthesis of acridone-tagged DNA.

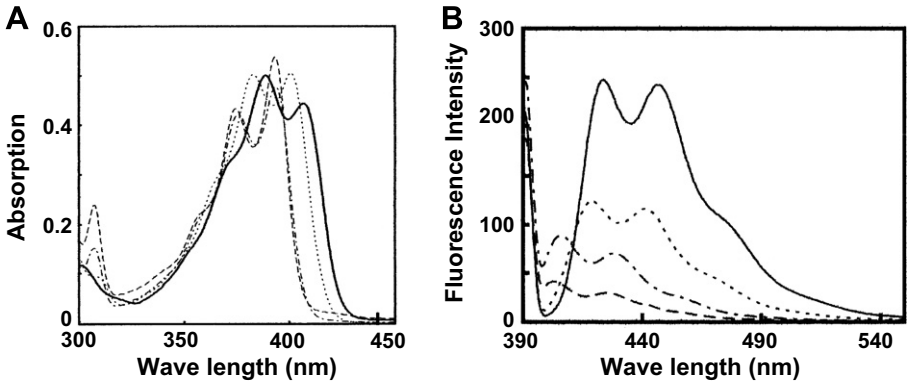


Figure 1. Absorption and emission spectra of acridone carboxylic acid. (A) Absorption spectra of acridone carboxylic acid (0.1 mM) in water (solid line), methanol (thin broken line), acetonitrile (one-dot broken line), and tetrahydrofuran (bold broken line). (B) Emission spectra of acridone carboxylic acid (0.5 μM) in water (solid line), methanol (thin broken line), acetonitrile (one-dot broken line), and tetrahydrofuran (bold broken line). Excitation at 388 nm.

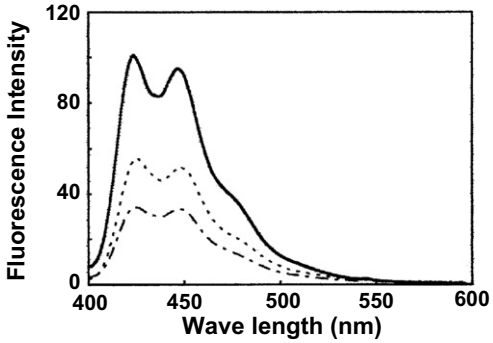
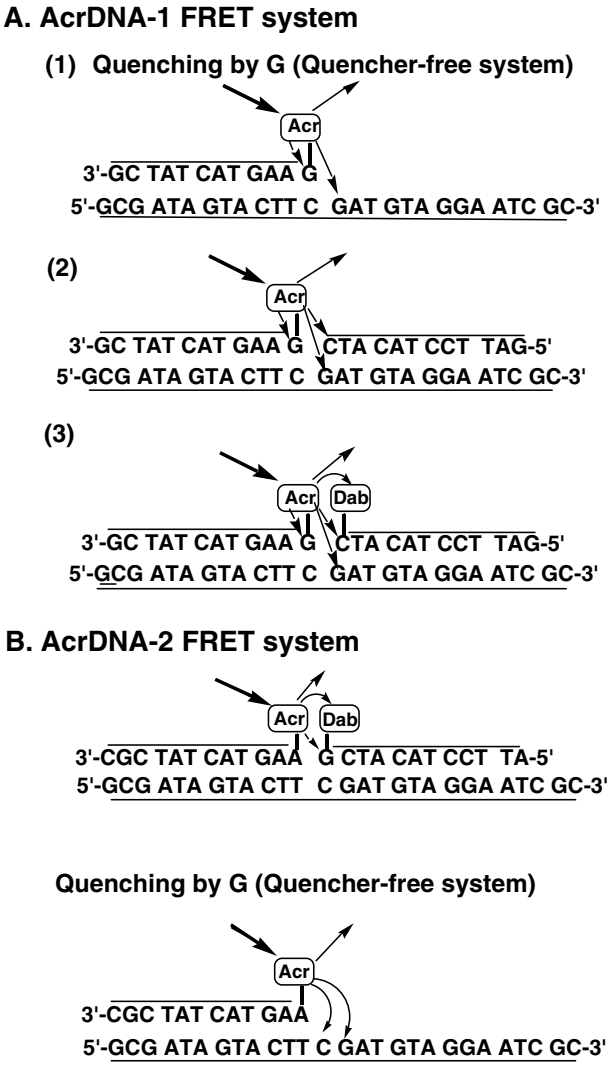


Figure 2. Emission spectra of acridone-tagged oligoDNAs. In the presence of 25-0 mM NaCl and 5 mM $MgCl_2$, 0.25 μM of AcrDNA-**1**, AcrDNA-**2**, or AcrDNA-**3** in 50 mM Tris–HCl (pH 7.6) was excited at 388 nm. One-dot broken line, AcrDNA-**1**; solid line, AcrDNA-**2**; broken line, AcrDNA-**3**.

found effect on the fluorescence intensity of acridone-tagged DNA. The acridone-quenching activity by flanking DNA bases is in the following order, G > C > A. The quenching by the DNA base likely occurs by energy transfer, although the precise mechanism of this fluorescence quenching is not clear. It has been reported that almost all fluorescent dyes are quenched most efficiently by the nucleobase guanine, followed by adenine, cytosine, and thymidine.[16,23] The high quenching efficiency of guanine is explained by the good electron-donating ability of guanine.[23] In the case of pyrene-labeled DNA, fluorescence quenching of pyrene is observed by the flanking G, C, and T through electron transfer, but not by A.[24]

2.4. FRET using acidone-tagged DNA and dabcyl-tagged DNA

To assess the efficiency of acridone as a donor for a FRET system coupled with dabcyl as a quencher, we designed a target DNA detection by nucleic acid hybridization using FRET in a homogeneous solution as a model system (Scheme 2). Three synthetic oligodeoxyribonucleotides were used for the system A; the first, **1** is attached with acridone at the 5′-end (AcrDNA-**1**), the second is labeled with dabcyl at the 3′-end (DabDNA-**5**), and the third that is the model target DNA (DNA-**4**) for which the sequence is complementary to AcrDNA-**1** and DabDNA-**5**. The three DNA are expected to form a tripartite duplex structure in which acridone and dabcyl are situated in close proximity, leading to efficient fluorescence quenching. The ratio of AcrDNA-**1**, DNA-**4**, and DabDNA-**5** was set to 1:1:1 and 1:2:3. We further examined the fluorescence properties of AcrDNA-**1** and DNA-**4** in order to gain information regarding the quenching effect of the noncovalent neighboring DNA base on the target DNA-**4**. We also studied the fluorescence of a tripartite duplex composed of AcrDNA-**1**, DNA-**4,** and DNA-**5** to investigate the quenching effect of the 3′-terminal base of DNA-**5**, which is devoid of the dabcyl moiety. Figure 3A shows the fluorescence



Scheme 2. FRET system composed of AcrDNA, DabDNA, and target DNA.

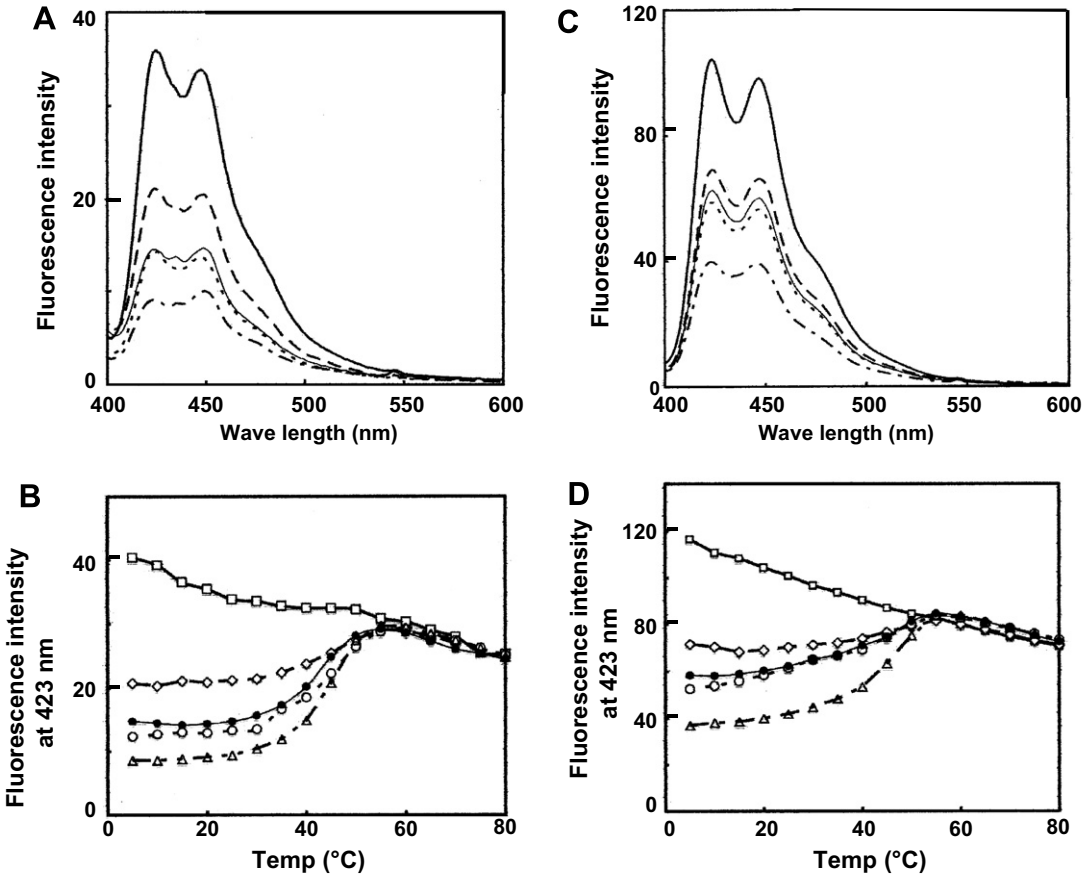


Figure 3. Fluorescence spectra of single-stranded and duplex acridone-tagged DNA. Fluorescence spectra were measured at 0.25 μM of AcrDNA in 50 mM Tris–HCl (pH 7.6) in the presence of 250 mM NaCl and 5 mM $MgCl_2$ by excitation at 388 nm. (A) Emission spectra of AcrDNA-**1** and duplexes at 5 °C. (B) Temperature-dependent change in peak fluorescence intensity (423 nm) of AcrDNA-1 and duplexes. Bold solid line and open square (a), AcrDNA-**1**; long broken line and open rhombic (b), AcrDNA-**1**:DNA-**4**; thin solid line and filled circle (c), AcrDNA-**1**:DNA-**4**:DNA-**5** (1:1:1); thin broken line and open circle (d), AcrDNA-**1**:DNA-**4**:DabDNA-**5** (1:1:1); one-dot broken line and open triangle (e), AcrDNA-**1**:DNA-**4**:DabDNA-**5** (1:2:3). (C) Emission spectra of AcrDNA-**2** and duplexes at 5 °C. (D) Temperature-dependent change in peak fluorescence intensity (423 nm) of AcrDNA-2 and duplexes. Bold solid line and open square, AcrDNA-**2**; long broken line and open rhombic, AcrDNA-**2**:DNA-**4**; thin solid line and filled circle, AcrDNA-**2**:DNA-**4**:DNA-**6** (1:1:1); thin broken line and open circle, AcrDNA-**2**:DNA-**4**:DabDNA-**6** (1:1:1); one-dot broken line and open triangle, AcrDNA-**2**:DNA-**4**:DabDNA-**6** (1:2:3).

spectra of AcrDNA-**1** (a), AcrDNA-**1**, and DNA-**4** in a 1:1 ratio (b), AcrDNA-**1**, DNA-**4**, and DNA-**5** in a 1:1:1 ratio (c), AcrDNA-**1**, DNA-**4**, and DabDNA-**5** in a 1:1:1 ratio (d), and AcrDNA-**1**, DNA-**4**, and DabDNA-**5** in a 1:2:3 ratio (e) at 5 °C at the same AcrDNA-**1** concentration. Duplex formation between AcrDNA-**1** and DNA-**4** decreased the fluorescence intensity by half, probably due to quenching by the proximate guanine on the DNA-**4**, as shown in Scheme 2A-(1). The addition of DNA-**5** to the AcrDNA-**1**:DNA-**4** duplex resulted in a further decrease in fluorescence to 36% of single-stranded AcrDNA-**1**. Cytosine at the 3′-terminal of DNA-**5** is brought close to the acridone of AcrDNA-**1** by tripartite duplex formation, and quenches the fluorescence, as shown in Scheme 2A-(2). The hydrophobic microenvironment surrounding the acridone moiety formed by tripartite duplex formation could also contribute to the quenching of the fluorophore. The fluorescence intensity of the AcrDNA-**1**:DNA-**4**:DabDNA-**5** (1:1:1) tripartite duplex was 30% of that of single-stranded AcrDNA-**1** (Scheme 2A-(3)). The increase in quenching in the presence of 3′-terminal dabcyl in DNA-**5** is only 6% compared to the same duplex without the dabcyl moiety. However, in the case of AcrDNA-**1**, DNA-**4**, and DabDNA-**5** in 1:2:3 molar ratio, the quenching was enhanced and fluorescence intensity decreased to 20%. Under the 1:2:3 ratio setting, the majority of AcrDNA-**1** could completely form a tripartite duplex with DNA-**4** and DabDNA-**5** to enhance the fluorescence quenching. Figure 3B illustrates the temperature-dependent fluorescence change at 423 nm in the five cases described above, (a), (b), (c), (d), and (e). The fluorescence of AcrDNA-**1** decreased gradually with increasing temperature. In contrast, AcrDNA-**1** in the duplex forms, (b), (c), (d), and (e), showed the typical sigmoid melting behavior of duplex DNA. The melting temperatures are estimated to be around 43 °C. AcrDNA-**2** and the same target DNA-**4** with the corresponding DabDNA-**6** or DNA-**6** showed similar fluorescence behaviors as AcrDNA-**1**, as shown in Figure 3C and D (Scheme 2B), although AcrDNA-**2** has stronger fluorescence emissions than AcrDNA-**1** because of quenching by flanking guanine at the 5′-terminal of DNA-**1**, as mentioned above. The results demonstrate that a FRET system using acridone as a fluorescence donor and dabcyl as a quencher can be applied as a probe for a homogeneous hybridization assay of the DNA and other assays for biological molecules, although there is a considerable amount of quenching by nucleotide bases, especially by guanine. On the other hand, strong quenching of acridone emissions by guanine may be used for the quencher-free probe for DNA detection, depending on the target DNA sequence.

In order to compare the efficiency of resonance energy transfer of the acridone–dabcyl pair with the conventional donor–quencher pair, we examined quenching of emissions using fluorescein-tagged DNA, dabcyl-tagged DNA, and target DNA with the same DNA sequence as used with acridone-tagged DNA. The combination of fluorescein and dabcyl proved to be a good FRET pair and

has been employed for several studies of molecular beacons[17,18] and aptamer beacons for detection of target DNA and protein.[19–21] FluDNA-**1** has the same DNA sequence and amino-linker at the 5′-terminal position as AcrDNA-**1**. Fluorescence spectra of FluDNA-**1** (a), FluDNA-**1**, and DNA-**5** in a 1:1 ratio (b), FluDNA-**1**, DNA-**4**, and DNA-**5** in a 1:1:1 ratio (c), FluDNA-**1**, DNA-**4**, and DabDNA-**5** in a 1:1:1 ratio (d), and FluDNA-**1**, DNA-**4**, and DabDNA-**5** in a 1:2:3 ratio (e) at 5 °C are shown in Figure 4A. The peak fluorescence intensity of FluDNA-**1** at 515 nm decreased to 80%, 79%, 63%, and 54% in (b), (c), (d), and (e) systems, respectively, compared to that of single-stranded FluDNA-**1** (a). The results show that a proximate nucleotide base does not effectively quench the emissions of fluorescein as compared with those of acridone. In particular, quenching by cytosine in the 5′-terminal of DNA-**5** was marginal. However, significant amounts of quenching by dabcyl occurred, in accordance with previous reports.[16,17] The fluorescence emission of the fluorescein–dabcyl pair in the 1:1:1 system of FluDNA-**1**, DNA-**4**, and DabDNA-**5** was 63% to that of the single-stranded FluDNA-**1**. On the other hand, the emission of the acridone–dabcyl pair in the same system decreased to 30% to that of the single-stranded AcrDNA-**1**, although the quenching of the emission of acridone took place mainly by proximate guanine base in addition by dabcyl. Figure 4B shows the melting behavior of FluDNA-**1** (a) and the duplexes formed from FluDNA-**1**, (b), (c), (d), and (e), were monitored at a peak fluorescence intensity of 515 nm. The melting temperature of the FluDNA-**1** duplex in the (b) system is 37 °C, which is several degrees lower than that of the corresponding AcrDNA-**1** duplexes. Tricyclic aromatic acridone likely enhances the hybridization ability of DNA compared with fluorescein. Figure 4C shows the fluorescence spectra of FluDNA-**2** and duplexes composed from FluDNA-**2**. FluDNA-2 has the same DNA sequence and linker as AcrDNA-2. The fluorescence intensity of FluDNA-**2** is stronger than that of FluDNA-**1** at the same concentration in the same order as that of the corresponding AcrDNA. The flanking guanine at the 5′-terminal of DNA-**1** exhibited substantial quenching of both acridone and fluorescein. The melting behaviors of the duplexes formed from FluDNA-**2** are shown in Figure 4D. The melting temperature of the FluDNA-**2** duplex is also lower than that of the corresponding duplexes formed from the AcrDNA-2 duplex. In comparison with the acridone- and fluorescein-tagged DNA with same DNA sequences, the acridone-tagged DNA showed smaller fluorescence intensity than the fluorescein-tagged DNA. The fluorescence intensity or the brightness of a dye depends on both its quantum yield and absorption coefficient of the dye at excitation wavelength. The absorption coefficient of acridone is much smaller than that of fluorescein at the excitation wavelength, and thus fluorescence intensity of the acridone-tagged DNA is smaller than that of the corresponding fluorescein-tagged DNA, although acridone has high quantum yield of the fluorescence.

The present results demonstrate that a combination of acridone- and dabcyl-tagged DNA can generate an easily detectable fluorescence change in the presence of target DNA in homogenous

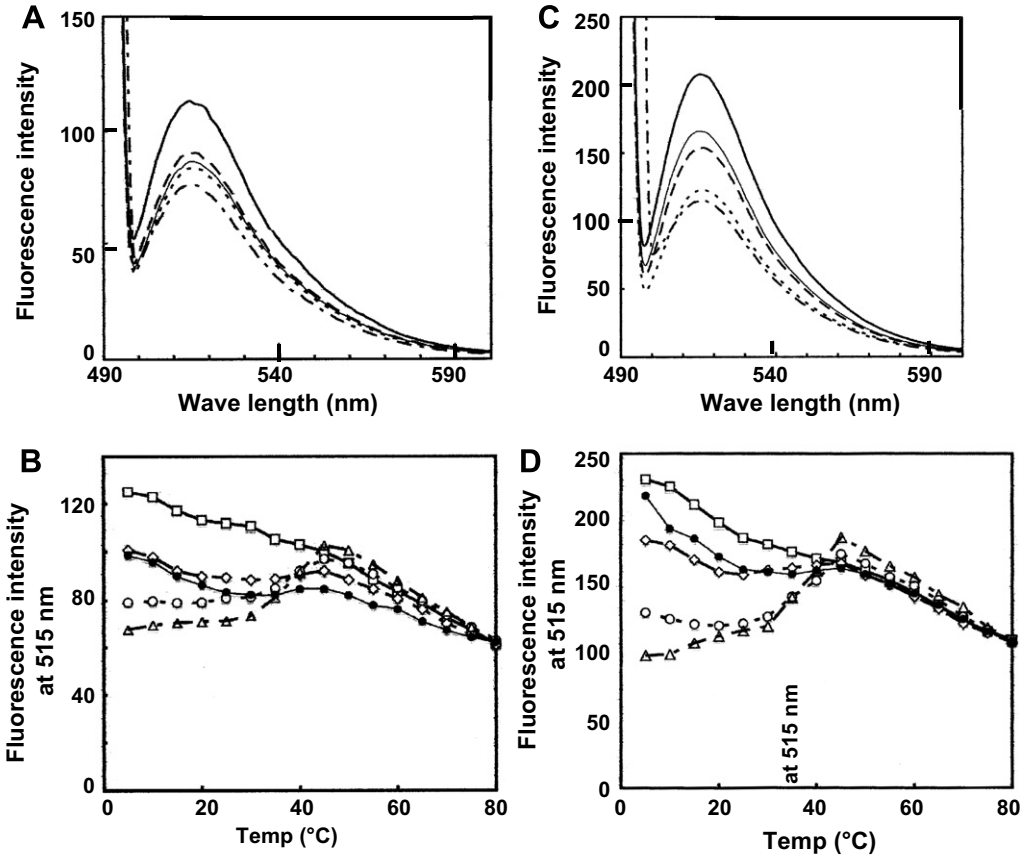


Figure 4. Fluorescence spectra of single-stranded and duplex fluorescein-tagged DNA. Fluorescence spectra were measured at 0.04 μM of FluDNA in 50 mM Tris–HCl (pH 7.6) in the presence of 250 mM NaCl and 5 mM $MgCl_2$ by excitation at 488 nm. (A) Emission spectra of FluDNA-**1** and duplexes at 5 °C. (B) Temperature-dependent change in the peak fluorescence intensity (515 nm) of FluDNA-1 and duplexes. Bold solid line and open square (a), FluDNA-**1**; long broken line and open rhombic (b), FluDNA-**1**:DNA-**4**; thin solid line and filled circle (c), FluDNA-**1**:DNA-**4**:DNA-**5** (1:1:1); thin broken line and open circle (d), FluDNA-**1**:DNA-**4**:DabDNA-**5** (1:1:1); one-dot broken line and open triangle (e), FluDNA-**1**:DNA-**4**:DabDNA-**5** (1:2:3). (C) Emission spectra of FluDNA-**2** and duplexes at 5 °C. (D) Temperature-dependent change in peak fluorescence intensity (515 nm) of FluDNA-2 and duplexes. Bold solid line and open square, FluDNA-**2**; long broken line and open rhombic, FluDNA-**2**:DNA-**4**; thin solid line and filled circle, FluDNA-**2**:DNA-**4**:DNA-**6** (1:1:1); thin broken line and open circle, FluDNA-**2**:DNA-**4**:DabDNA-**6** (1:1:1); one-dot broken line and open triangle, FluDNA-**2**:DNA-**4**:DabDNA-**6** (1:2:3).

solution. The change in the fluorescence intensity of the acridone–dabcyl pair is comparable to or somewhat larger than that of the fluorescein–dabcyl pair,[11,18] although the fluorescence intensity of the acridone-tagged DNA is smaller than that of the corresponding fluorescein-tagged DNA. The combination of acridone and dabcyl provides a new FRET pair and complements other fluorophore–quencher combinations, although the performance of the FRET composed of acridone and dabcyl DNA is not satisfactory and needs improvement, such as using acridone- and dabcyl-tagged DNA with a short and rigid linker to facilitate a closer interaction of the fluorophore and the quencher, or using another acridone derivative that has stronger fluorescence intensity and better spectral overlapping of acceptor's absorption and donor's emission spectra.

2.5. One-base mismatch DNA discrimination by acridone-tagged DNA at the C5 position of inside T

We further studied the photochemical and thermal melting behavior of DNA bearing acridone at the C5 position of thymidine via an aminohexyl linker in order to determine whether the acridone-tagged DNA can discriminate the single mismatch DNA. The sequences of acridone-tagged DNA (AcrDNA-**7**) and the complementary DNA with or without single base mismatch DNA (DNA-8A, DNA-8T, DNA-8C, and DNA-8G) are shown in Scheme 3. The mismatch base is present opposite to the acridone-tagged thymidine residue. Figure 5A shows the fluorescence spectra of the single-stranded and duplexes composed of AcrDNA-**7** and DNA-8A ~ G at 5 °C. The full-matched and G-T mismatched duplexes showed weak fluorescence intensity compared to the single-stranded DNA. The C-T mismatched duplex also showed a substantial decrease in fluorescence. In contrast, T-T mismatched duplex exhibited only a small decrease in fluorescence. Thus, the acridone-tagged DNA can discriminate T-T mismatch duplex and may be applicable as a probe for T-T mismatch DNA detection, although the discrimination ability is not satisfactory for the practical use of single-nucleotide polymorphisms typing. In the case of single-stranded DNA, the acridone moiety is surrounded by aqueous phase, and thus displays strong fluorescence emissions. When full-matched duplex of AcrDNA-**7** is formed, the acridone moiety could be located in the major groove or inside of the duplex by intercalation, in which the hydrophobic microenvironment decreases the acridone emissions. In the case of T-T mismatched duplex, the acridone moiety could be facing outside the duplex, where the aqueous hydrophilic environment enhances the fluorescence emissions of acridone. G and T can form a base-pair, although weak, thus the acridone moiety in the G-T mismatched duplex showed similar behavior as that in the full-matched DNA. Quenching of fluorescence by G likely occurred in the G-T mismatched duplex. In case of C-T mismatched duplex, T and C could not form a base-pair, although the acridone at the C5 position of T with a longer linker could be close to C in the mismatched DNA, and thus the acridone emission could be quenched by C. The observation that emissions of AcrDNA-**7** were decreased by full-matched DNA duplex formation is contradictory to the results of Saito et al., who reported that full-matched duplex of their acridone-labeled DNA exhibited stronger emissions than the single-stranded DNA[15] because the acridone fluorophore is extruded to the outside of the groove when the full-matched duplex is formed. The discrepancy of the observation between ours and theirs is likely derived from the differences in the acridone derivative, linker, and DNA base sequence. Previously we have shown that a substituent group at the C5 position of thymidine with a long flexible linker in the modified DNA is likely in the major groove when duplex is formed with the complementary DNA.[25] Acridone with the long and flexible linker in this acridone-tagged DNA could be also in the major groove when the full-matched duplex is formed, and thus emission of the acridone may be decreased. We used a long and flexible hexamethylenediamine linker, while Saito's group used a short and rigid linker to connect the acridone to the C8 position of adenine or the C5 position of uridine. Moreover, they introduced the acridone into the labeled DNA at the AT successive region, while we used mix sequenced DNA as a model system. Thus, there are some limitations to the use of acridone-labeled DNA as a probe for single-nucleotide polymorphism typing of any DNA sequences, because proximate guanine base has large

5′-CAT AGG AGA XGC CTA-3′ : AcrDNA-7
5′-CAT AGG AGA TGC CTA-3′ : DNA-7
3′-GTA TCC TCT ACG GAT-5′ : DNA-8A
3′-GTA TCC TCT TCG GAT-5′ : DNA-8T
3′-GTA TCC TCT CCG GAT-5′ : DNA-8C
3′-GTA TCC TCT GCG GAT-5′ : DNA-8G

Scheme 3. Oligonucleotides used for the DNA mismatch recognition by acridone-labeled DNA.

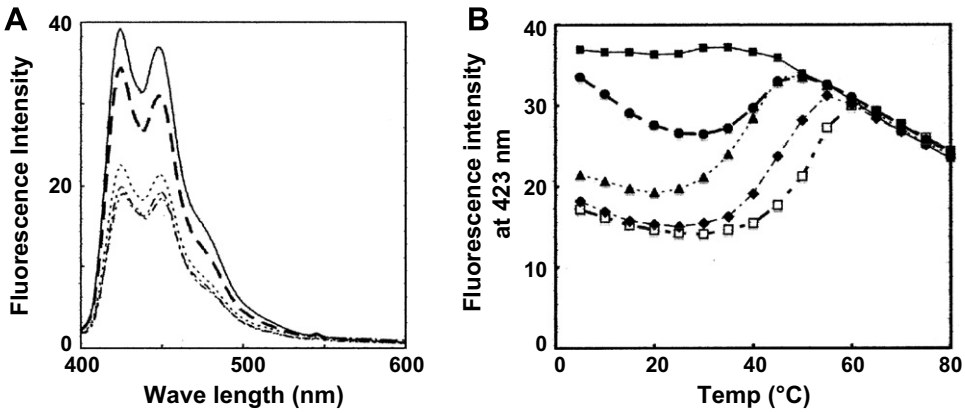


Figure 5. Fluorescence spectra of single-stranded and duplex forms of AcrDNA-**7**. In the presence of 150 mM NaCl, 0.25 μM of AcrDNA-7 in 10 mM sodium phosphate buffer (pH 7.0) was excited at 388 nm in the absence or presence of complementary DNA with either a full-match or a single mismatch base. (A) Emission spectra at 5 °C, thin solid line, AcrDNA-**7**; bold broken line, AcrDNA-**7**:DNA-**8T**; thin broken line, AcrDNA-**7**:DNA-**8C**; One-dot broken line, AcrDNA-**7**; DNA-**8G**; two-dot broken line, AcrDNA-**7**:DNA-**8A** (full-match). (B) Temperature-dependent change in fluorescence intensity at 423 nm: closed square, AcrDNA-**7**; closed circle, AcrDNA-**7**:DNA-**8T**; closed triangle, AcrDNA-**7**:DNA-**8C**; closed rhombic, AcrDNA-**7**;DNA-**8G**; open square, AcrDNA-**7**:DNA-**8A** (full-match).

Table 1
Melting temperature of AcrDNA-**7** duplex

DNA	T_m (°C)
Natural DNA duplex	49.8
AcrDNA-**7**: DNA-**8A**	45.9
AcrDNA-**7**: DNA-**8T**	37.0
AcrDNA-**7**: DNA-**8C**	36.3
AcrDNA-**7**: DNA-**8G**	42.2

T_m was estimated from the temperature-dependent fluorescence change at 0.25 μM DNA concentration except natural DNA duplex.

effect on the emission of acridone. Melting temperatures (T_m) of the full-matched and mismatched duplexes composed of AcrDNA-**7** estimated from the melting behavior of the duplexes (Fig. 5B) are listed in Table 1. The T_m values of the fully matched duplex (AcrDNA-**7**:DNA-**8A**) are slightly lower than that of the corresponding natural type DNA. The mismatched duplexes showed further lower T_m values, as expected.

In conclusion, our results regarding the fluorescence properties of acridone-tagged DNA demonstrate that acridone-labeled DNA can be applied as a fluorescent probe for the detection of specific DNA using FRET with a quencher such as dabcyl or guanine base in the DNA without an additional quencher, and may be applied to SNP detection. However, it should be noted that the emission of the acridone varies greatly depending on the hydrophobicity of the surrounding environment, and care should be taken in the design of the probe, including the linker, DNA sequence, and the fluorophore attachment site. On the other hand, the fluorescence property of acridone that shows strong change in the emission depending on the guanine base and on the hydrophobicity of the surrounding environment could make use of the acridone as a quencher-free probe for FRET system and special DNA detection.

3. Experimental

3.1. Analytical methods

UV spectra were taken with a Hitachi 3200 spectrometer. The UV-thermal denaturation behavior of the association complex of the DNA was measured with a Shimazu UV-2550 equipped with a thermal controller, and T_m values were determined with a TMSPC-8. Fluorescence spectra were recorded with a Shimazu RF-5300C equipped with a thermal controller. ESI mass spectra were taken with a Perkin Elmer Sciex API-100. NMR spectra were measured with a JEOL JMN-AL-300. MALDI-TOF mass spectra were taken with an ABI Voyager AXIMA-CF. High-pressure liquid chromatography (HPLC) on an ODS-silica gel column (4.6 mm × 250 mm) was carried out with a linear gradient elution (0–25%) of acetonitrile in 50 mM triethylammonium acetate (pH 7.0) over 35 min at a flow rate of 1.0 ml/min. Gel electrophoresis was carried out on 20% polyacrylamide gels containing 7 M urea, and bands on the gel were imaged with a Molecular Imager® FX (Bio-Rad) equipped with an external laser module. The concentration of each oligodeoxyribonucleotide was determined spectrophotometrically using the molar coefficient estimated from the published method.[26]

3.2. Materials

Acridonyl acetic acid (**1**) was prepared by the published procedure.[14,22] Fluorescein isothiocyanate (FITC) was obtained from Wako Chemical Co. Oligodeoxyribonucleotides with a 5′-terminal amino group via C6-linker, [5′-NH_2-C_6-GAA GTA CTA TCG-3′ (DNA-**1**), 5′-NH_2-C_6-AAG TAC TAT CGC-3′ (DNA-**2**) and 5′-NH_2-C_6-CAT AGG AGA TGC CTA-3′ (DNA-**3**)], 5′-GCG ATA GTA CTT CGA TGT AGG AAT CGC-3′ (DNA-**4**), 3′-end dabcyl-tagged oligodeoxyribonucleotides, [5′-GAT TCC TAC ATC-3′-dabcyl (DabDNA-**5**) and 5′-ATT CCT ACA TCG-3′-dabcyl (DabDNA-**6**)], 5′-GAT TCC TAC ATC-3′ (DNA-**5**), and 5′-ATT CCT ACA TCG-3′ (DNA-**6**) were purchased from Nippon Bio Service (Tokyo, Japan). The modified DNA containing an aminohexyl linker at the C5-position of 2′-deoxythymidine (5′-CAT AGG AGA T^{NH2}GC CTA-3′, DNA-**7**) was prepared by our published procedure using an automated DNA synthesizer.[27] Oligodeoxyribonucleotides, 5′-CAT AGG AGA TGC CTA-3′ (DNA-**7**′), 5′-GTA TCC TCT ACG GAT-3′ (DNA-**8A**), 5′-GTA TCC TCT TCG GAT-3′ (DNA-**8T**), 5′-GTA TCC TCT CCG GAT-3′ (DNA-**8C**), and 5′-GTA TCC TCT GCG GAT-3′ (DNA-**8G**), were obtained from Hokkaido System Science Co. All other chemicals used in this study were reagent grade and were used without further purification.

3.3. Synthesis of acridone-tagged DNA

3.3.1. *N*-Hydroxysuccinimide ester of acridone acetic acid (2)

A mixture of acridone acetic acid **1** (127 mg, 0.50 mmol), *N*-hydroxysuccinimide (56 mg, 0.75 mmol), and dicyclohexyl carbodiimide (155 mg, 0.75 mmol) in dry DMF (10 ml) was stirred at room temperature for 10 h under nitrogen. After the reaction, the solvent was removed in vacuo, and ethyl acetate containing 10% DMF was added to the residue. The resulting precipitate was removed by filtration, and the filtrate was evaporated under reduced pressure and washed with dry ether several times to give *N*-hydroxysuccinimide ester of acridone acetic acid **2** as a yellow powder, (155 mg, 0.44 mmol, 89%). ESI-MS: *m*/*z*, 349.3, calcd, for $[M-H]^+$, 349.1. The product **2** was used for the synthesis of acridone-tagged DNA without further purification.

3.3.2. Acridone-tagged DNA-1 (AcrDNA-1)

A solution of **2** (7 μmol/mL) in DMF was prepared from **2** (1 mg, 2.9 μmol) and dry DMF (400 μl), and 215 μl of the solution (1.7 μmol of **2**) was added to the mixture of DNA-**1** (4.3 OD_{260}, 34 nmol) in distilled water (123 μl) and saturated sodium hydrogen carbonate solution (48 μl). The reaction mixture was stirred at room temperature, and the progress of the reaction was checked by HPLC after 2 and 4 h. After 5 h, the solution was evaporated in vacuo, and the residue was dissolved in distilled water. Ethanol was added to the solution for precipitation, which was purified by HPLC to give the purified AcrDNA-**1** in 13% yield, (0.79 OD_{260}). MALDI-mass *m*/*z*: found 4084.9; calcd for $[M+H]^+$ 4084.9. The yield was calculated from the estimation that the molar absorption coefficient of AcrDNA-**1** is the sum of that of acridone (ε_{260} = 50,000) and DNA-**1** (ε_{260} = 124,000). Estimated ε_{260} of AcrDNA-**1** was 174,000.

AcrDNA-**2** was synthesized by the same method as that of AcrDNA-**1** from DNA-**2** (4.0 OD_{260}, 33 nmol) and **2** in 14% yield (0.78 OD_{260}). MALDI-mass *m*/*z*: found 4045; calcd for $[M+H]^+$ 4044. Estimated ε_{260} values of DNA-**2** and AcrDNA-**2** were 120,000 and 170,000, respectively.

AcrDNA-**3** was obtained from DNA-**3** (3.0 OD_{260}, 20 nmol) and **2** by a similar method with slight modification. In brief, a mixture of acridone acetic acid **1** (2.1 mg, 7.9 μmol), *N*-hydroxysuccinimide (1.2 mg, 9.5 μmol), and dicyclohexyl carbodiimide (2.4 mg, 10 μmol) in dry DMF (1 ml) was stirred at room temperature for 5 h. Next, 125 μl of the solution (1.0 μmol of **2**) was added directly to the solution of DNA-**3** (3.0 OD_{260}, 20 nmol) in distilled water (230 μl) and 1 M sodium hydrogen carbonate solution (78 μl), and was stirred at room temperature for 8 h. The product was isolated as described above in 15% yield (0.61 OD_{260}). MALDI-mass *m*/*z*: found 5017.0; calcd for $[M+H]^+$ 5016.3. Estimated ε_{260} values of DNA-**3** and AcrDNA-**3**were 151,000 and 201,000, respectively.

AcrDNA-**7** was obtained from DNA-**7** and **2** by a similar method, as described above. A mixture of acridone acetic acid **1**(2.1 mg, 7.9 μmol), *N*-hydroxysuccinimide (1.2 mg, 9.5 μmol), and

dicyclohexyl carbodiimide (2.4 mg, 10 μmol) in dry DMF (1 ml) was stirred at room temperature for 15 h, and 208 μl of the solution (1.6 μmol of **2**) was added directly to the solution of DNA-**7** (5.0 OD_{260}, 33 nmol) in distilled water (160 μl) and 1 M sodium hydrogen carbonate solution (63 μl), and was stirred at room temperature for 9 h. The product was isolated as described above in 52% yield (3.5 OD_{260}). MALDI-mass *m*/*z*: found 4979.0; calcd for $[M+H]^+$ 4979.3. Estimated ε_{260} values of DNA-**7** and AcrDNA-**7**were 153,000 and 203,000, respectively.

3.4. Synthesis of fluorescein-tagged DNA

3.4.1. Fluorescein-tagged DNA-1 (FluDNA-1)

A solution of FITC (2 mg, 5 μmol) in dry DMF (70 μl) was prepared, and 10 μl of the solution (0.7 μmol of **2**) was added to the mixture of DNA-**1** (2.0 OD_{260}, 16 nmol) in 1 M sodium hydrogen carbonate solution (20 μl). The reaction mixture was stirred at room temperature with an aluminum foil cover to protect from light, and the progress of the reaction was checked by HPLC. After 8 h, 30% aqueous ammonia (10 μl) was added to the reaction mixture to stop the solution and was evaporated in vacuo to give an oily residue that was dissolved in distilled water. The solution was subjected to gel filtration column chromatography on Sephadex G-15 (15 × 140 mm) to remove the low-molecular weight compounds from FITC. The appropriate fractions were collected, condensed under reduced pressure, and purified by HPLC to give purified FluDNA-**1** in 55% yield, (1.2 OD_{260}). The yield was calculated from the estimation that the molar absorption coefficient of FluDNA-**1** is the sum of that of fluorescein (ε_{260} = 21,000) and DNA-**1** (ε_{260} = 124,000). MALDI-mass *m*/*z*: found 4239.9; calcd for $[M+H]^+$ 4239.0. Estimated ε_{260} value of FluDNA-**1** was 145,000.

FluDNA**2** was synthesized by the same method as that of FluDNA-**1** from DNA-**2** (2.0 OD_{260}, 17 nmol) and FITC in 34% yield (0.8 OD_{260}). MALDI-mass *m*/*z*: found 4200; calcd for $[M+H]^+$ 4198. Estimated ε_{260} value of FluDNA-**2** was 140,000.

The molar coefficient of dabcyl-tagged DNA was estimated from the sum of that of dabcyl (ε_{260} = 15,600) and DNA. The estimated molar coefficients of DabDNA5, DabDNA6, DNA4, DNA5, and DNA6 were 128,000, 129, 000, 267,000, 112,000, and 113,000, respectively.

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