Detection of methylcytosine by DNA photoligation *via* hydrophobic interaction of the alkyl group†

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Received 11th March 2009, Accepted 6th May 2009
First published as an Advance Article on the web 11th June 2009
DOI: 10.1039/b904941j

We report the nonenzymatic detection of 5-methylcytosine by using template-directed photoligation through 5-vinyl-2′-deoxyuridine derivatives with high selectivity. In this paper, we propose a new detection method by using hydrophobic interaction. The photoligation yield of the 5-methylcytosine case was approximately 5.6-fold higher than that in the case of cytosine.

Introduction

At the chemical level, gene silencing requires the selective methylation of the 5-position of cytidines in DNA, and this established method is known as the epigenetic control of gene function. For example, 5-methylcytosine (^{m}C) appears to be involved in various biologically important processes occurring at the cellular level, and plays a role in the regulation of gene expression, genomic imprinting, cell differentiation and tumorigenesis.[1] Technological advancement in DNA methylation analysis is an important and ongoing endeavor of epigenetic research. However, the epigenetic information in methylated DNA is lost upon PCR or subcloning as consecutive replication rounds result in the methylation pattern of the host strain.[2,3] Therefore, various methods have been developed for detecting ^{m}C based on chemical and enzymatic concepts.[4–13] Each method has advantages and disadvantages, most of which become evident by examining their specificity, resolution, sensitivity and potential artifacts. One such method is the reversible DNA photoligation method. The DNA template-directed reversible photoligation proceeds *via* [2 + 2] cycloaddition between the double bond of the 5-carboxyvinyl-2′-deoxyuridine side chain and the C5-C6 double bond of pyrimidine.[14] We recently reported a method for selective detection of 5-methylcytosine *via* DNA photoligation using 5-cyanovinyl-2′-deoxyuridine (^{C}U).[14] Significantly, 5-methylcytosine in the target sequence yielded ligated product, with a measured ratio of ligation yield that was 9.8-fold higher than the cytosine case. Although the photochemical ligation approach includes many advantages such as not needing additional reagents, there were disadvantages to using side-reactions for detecting methylcytosine such as photo-crosslinking, which is the connection of photosensitive DNA to the template DNA. Here we report a detection method of ^{m}C by using DNA photoligation *via* other photosensitive nucleosides. As shown in Fig. 1, our strategy is based on the hydrophobic interaction between the methyl group at the C5 position of the cytosine and the alkyl residue of the vinyluridine derivatives. We consider that the ligation yield was increased by stacking the vinyl group and the C5-C6 double bond to be promoted in the presence of a methyl group. Cytosine is expected to produce a low ligation yield as compared to methylcytosine because of low hydrophobicity in the absence of a methyl group. We propose that our method can reuse photosensitive DNA and methylated DNA samples because the photoligation reactions are reversible.

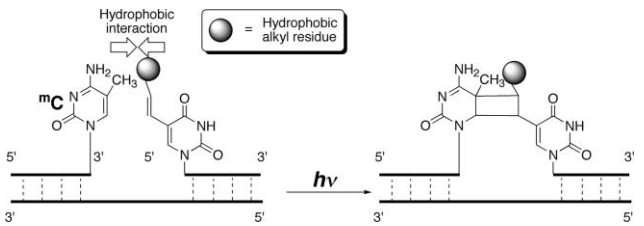

Fig. 1 Strategy for 5-methylcytosine detecting method by photoligation *via* hydrophobic interaction of each vinyluridine derivative.

Firstly, we synthesized 5-vinyl-2′-deoxyuridine (^{V}U) and three kinds of ^{V}U derivatives as photosensitive nucleosides. Each of the ^{V}U derivatives contains hydrophobic residue, pentyl, cyclohexyl and *tert*-butyl (Fig. 2).

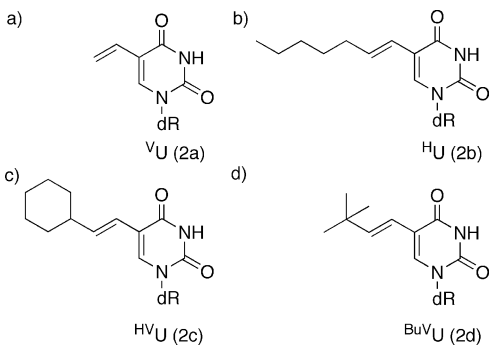

Fig. 2 Structure of 5-vinyl uracil (1a), 5-heptenyl uracil (1b), 5-cyclohexyl vinyl uracil (1c) and 5-*t*-butyl vinyl uracil (1d).

Results and discussion

The synthesis of the photosensitive nucleosides and the corresponding phosphoramidite building block of ^{V}U, 5-heptenyl-2′-deoxyuridine (^{H}U), 5-cyclohexyl vinyl-2′-deoxyuridine (^{HV}U)

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† Electronic supplementary information (ESI) available: Experimental details. See http://dx.doi.org/10.1039/b904941j/

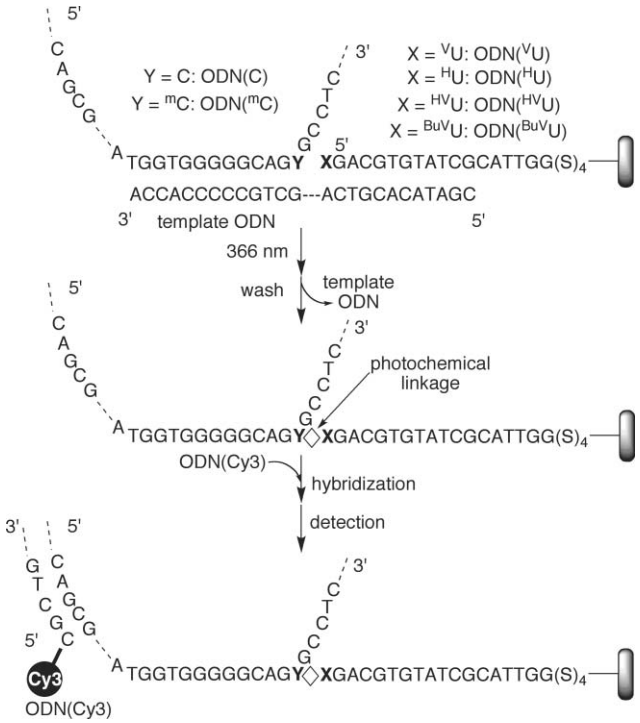
Table 1 ODN sequences used in this study

	Sequence (5′-3′)[a,b]
ODN(^{V}U)	^{V}UGACGTGTATCGCATTGGSSSSNH$_2$
ODN(^{H}U)	^{H}UGACGTGTATCGCATTGGSSSSNH$_2$
ODN(^{HV}U)	^{HV}UGACGTGTATCGCATTGGSSSSNH$_2$
ODN(^{BuV}U)	^{BuV}UGACGTGTATCGCATTGGSSSSNH$_2$
ODN(C)	GCCCCAGCTGCTCACCATCGCTATCTGAG CAGCGCTCATGGTGGGGGCAG**C**GCCTCA CAACCTCCGTCATGTGCTGTGACTGCTTG TAGATGGCCATGGC
ODN(^{m}C)	GCCCCAGCTGCTCACCATCGCTATCTGAG CAGCGCTCATGGTGGGGGCAG**^{m}C**GCCTCA CAACCTCCGTCATGTGCTGTGACTGCTTG TAGATGGCCATGGC
template ODN	CGATACACGTCAGCTGCCCCCACCA
ODN(Cy3)	Cy3-CGCTGCTCAGATAGC

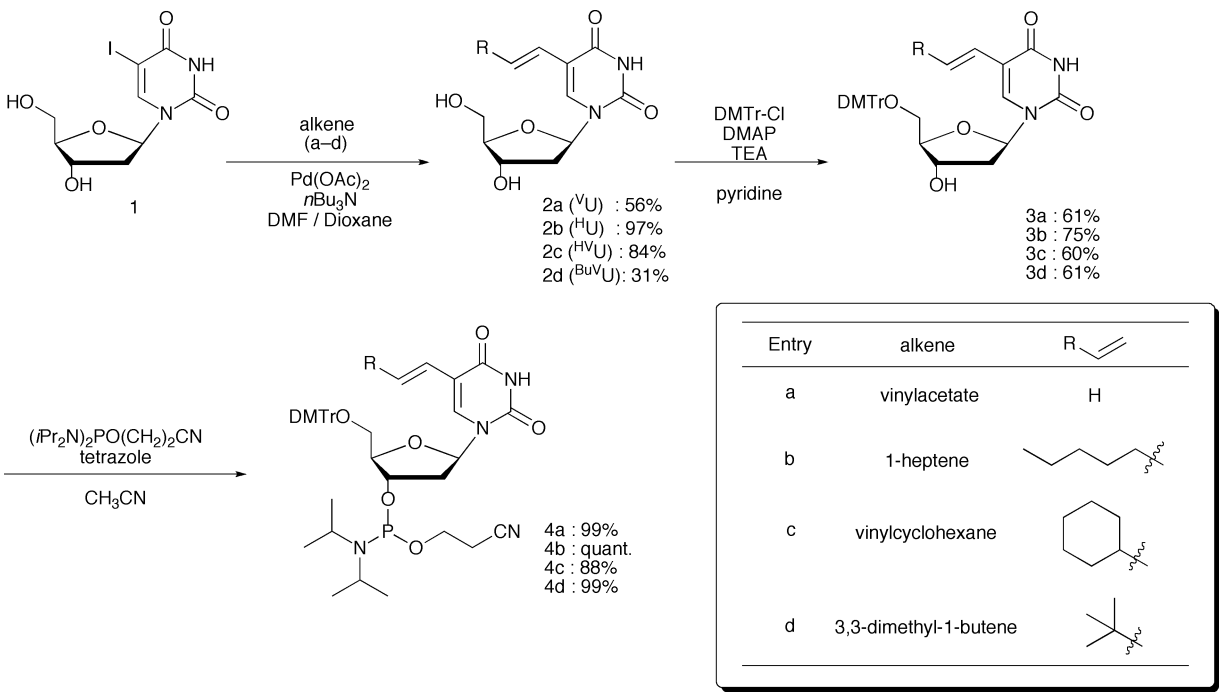
[a] Bold characters indicate the methylation status. [b] S corresponds to a hexa(ethylene glycol) linker fragment.

and 5-*t*-butylvinyl-2′-deoxyuridine (^{BuV}U) as well as the synthesis of corresponding oligodeoxynucleotide followed standard routes in DNA-chemistry.[15] ^{V}U was synthesized from 5-iodo-2′-deoxyuridine; the scheme has been reported previously.[14a] The synthesis of three kinds of ^{V}U derivatives is shown below. 5-Iodo-2′-deoxyuridine was converted to **2a-d** by the Heck reaction using various alkenes. **2a-d** was dimethoxytritylated and transferred to the corresponding cyanoethyl phosphoramidite using a conventional method (Scheme 1). The modified ODN was synthesized on an ABI 3400 DNA synthesizer. Incorporation of ^{V}U derivatives ODNs into the ODN was confirmed by enzymatic digestion and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (see ESI†). The ODNs used in this study are summarized in Table 1.

To demonstrate that template-directed photoligation by using each photosensitive ODN (Table 1) could be incorporated into platforms suitable for DNA chip technologies, we constructed a DNA chip by attaching amino-labeled ODN containing 5-vinyl-2′-deoxyuridine derivative, onto the aldehyde-modified glass surface (Scheme 2). We determined the feasibility



Scheme 2 Conceptual scheme showing how the target is detected by photoligation.



Scheme 1 The route for the synthesis of photosensitive nucleosides containing hydrophobic residue.

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of the template-directed photoligation through photosensitive ODN on a DNA chip. We prepared 60 mer DNA strands, 5′-d(...GGGGGCAG**X**GCCTCACAACC...)-3′, which contained a methylation hotspot (X = ^{m}C or C) at codon 175 in exon 5 of the p53 gene.[16] A glass chip spotted with 2 μM target ODN(C) or ODN(^{m}C) and template ODN, was irradiated at 366 nm for 60 min in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride. After the chip had been washed with deionized water at 98 °C for 5 min, Cy3-containing ODN(Cy3) conjugate was added to the surface, and the chip was washed twice in PBS. Fluorescence signals were detected on a microarray scanner. As shown in Fig. 3A, we measured the strong fluorescence signal of the photoligated product with the ^{m}C case by using ODN(^{H}U). The results show that a ^{m}C-containing ODN(^{m}C) yielded a highly photoligated product, with a measured fluorescence signal that was 5.6-fold higher than the C-containing ODN(C) case (Fig. 3B).

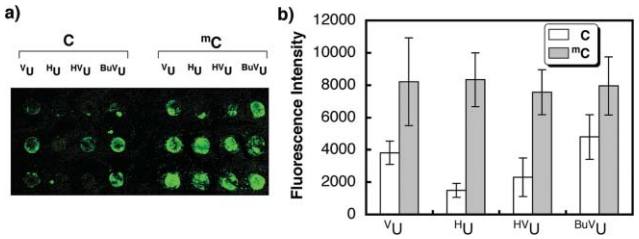

Fig. 3 (a) Fluorescence images acquired on a microarray scanner for the product of photoligation on cytosine and 5-methylcytosine target DNAs. (b) Selectivity for different photosensitive nucleotide conditions. The data points represent the average of three experimental runs by irradiation at 366 nm for 1 h.

We performed UV melting studies using model sequence 15 mer of the P53 gene (see ESI†) according to disclosed differences of reactivity of each vinyluridine derivative. As shown in Fig. 4 and Table 2, the ΔT_m values of the ODN containing ^{H}U were the highest of all combinations (ΔT_m = 3.4 °C). In this case, the ΔT_m values are defined by deducting the T_m value of the cytosine case from the methylcytosine case. We confirmed improvement of the ratio (^{m}C/C) corresponding to the rise of the ΔT_m value (Fig. 4). As a result, we suggested that photoligation is promoted by the thermal stability of methylcytosine and vinyluridine derivatives. Additionally, we considered that the balance of values between enthalpy and entholopy obtained by the van't Hoff plot would seem to explain the differences of reactivity (see ESI†). These results suggest that the hydrophobicity of photosensitive

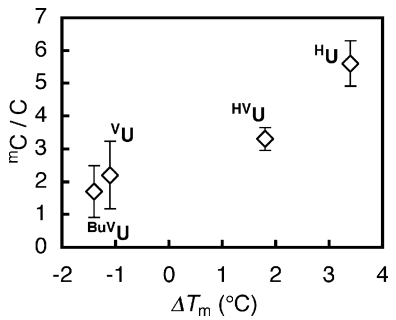

Fig. 4 The dependence of the fluorescent intensity ratio (^{m}C/C) on the ΔT_m values. ΔT_m values are defined by deducting the T_m value of the cytosine case from the methylcytosine case.

Table 2

	^{m}C /C[a]	ΔT_m[b,c]
^{V}U	2.2	−1.1 °C
^{H}U	5.6	3.4 °C
^{HV}U	3.3	1.8 °C
^{BuV}U	1.7	−1.4 °C

[a] Values of the ratio of the methylcytosine case to the cytosine case to be calculated from fluorescent intensity. [b] ΔT_m values are defined by deducting the T_m value of the cytosine case from the methylcytosine case. [c] Conditions: 100 mM NaCl, 50 mM Na cacodylate buffer (pH 7.0), 1.0 μM each strand.

nucleosides such as ^{H}U is able to detect the methyl group at the C5 position of the cytosine. We previously reported methylcytosine detection using 5-cyanovinyl-2′-deoxyuridine, which depended on the hydrophobicity and planarity of the cyanovinyl group.[17] We considered that the direction of the photoreaction was changed by the micro-environment such as the hydrophobic interaction and intercalation in the DNA strands by the presence/absence of the methyl group at cytosine C5. However, no side reaction was observed by using ^{H}U in this report. Therefore, we speculated that the difference in reactivity was caused by the heptenyl group of ^{H}U being bulkier than the cyanovinyl group.

To confirm the photosensibility of the ligation process and reuse of methylated DNA, irradiation of the photoligated product at 312 nm was examined. As a result, rapid disappearance of ligated ODN was observed to revert to ^{m}C-containing ODN and photosensitive ODN by 312 nm irradiation (see ESI†).

Conclusions

In conclusion, we synthesized several vinyluridine derivatives of which the nucleosides have the property of hydrophobicity. Then, we demonstrated a 5-methylcytosine detecting system by using a photoligation method through these vinyluridine derivatives. Significantly, 5-methylcytosine in the target sequence yielded ligated product, with a measured ratio of ligation yield that was 5.6-fold higher than the cytosine case. We considered that the differences were obtained by the thermal stability of methylcytosine and vinyluridine derivatives. Epigenetic research could not use such small samples because of the methylated DNA that is lost in PCR. Although we consider the reuse of methylated samples to be important, reuse of the sample was difficult in the case of using the ^{C}U method. Additionally, the photoligated oligonucleotides were quantitatively reverted to the original oligonucleotides 312 nm irradiation. Thus, these results suggest that our method can reuse photosensitive DNA and methylated DNA samples because the photoligation reaction is reversible.

Experimental section

General

Tetrazole was purchased from GLEN RESEARCH. The reagents for the DNA synthesizer such as I_2 solution (I_2/H_2O/pyridine/tetrahydrofuran, 3:2:19:76), A-, G-, C-, and T-β-cyanoethyl phosphoroamidites were also purchased from GLEN RESEARCH. Other reagents were purchased at the highest commercial quality and used without further purification

unless otherwise stated. Microwave reactions were conducted using the CEM Discover as a focused microwave unit. Calf intestine alkaline phosphatase (AP) (1,500 units) was purchased from Promega and Roch. Nuclease P1 (500 units) was purchased from Yamasa. Reactions were monitored on TLC plates precoated with Merck silica gel 60 F_{254}. Kanto Chemical Silica Gel 60 N was used for silica gel column chromatography. ODNs were synthesized on an Applied Biosystems 3400 DNA Synthesizer. Reverse phase HPLC was performed on a Cosmosil 5C_{18}AR-II (Nacalai Tesque) column (4.6 × 150 mm) or a CHEMCOBOND 5-ODS-H (Chemco) column (4.6 × 150 mm) with a JASCO PU-980, HG-980–31, DG-980–50 system equipped with a JASCO UV 970 spectrometer at 260 nm. Irradiation was performed by a transilluminator (Funakoshi TR-366 nm). Mass spectra were recorded on a Voyager-DE PRO-SF, Applied Biosystems.

Preparation of 5-heptenyl-2′-deoxyuridine (2b). 5-Iodo-2′-deoxyuridine (500 mg, 1.41 mmol) was dissolved in DMF in a microwave tube with a stirring bar. To this was added palladium (II) acetate (33 mg, 0.15 mmol), tri-*n*-butylamine (340 μl, 1.41 mmol) and 1-heptene (5 ml, 3.53 mmol). The reaction tube was sealed and reacted in a microwave reactor for 20 min at 100 °C with continuous stirring. The reaction mixture was filtered to remove the resulting precipitate, and extracted with EtOAc (20 ml x 3) and water (30 ml). The organic layer was collected, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under reduced pressure. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH 9:1) to afford 2b (326 mg, 1.36 mmol, 97%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.58 (s, 1H, H-C(6)); 6.21–6.13 (m, 1H, H-C(1′)); 6.04 (d, 1H, J= 15.9, vinylic H); 5.54–5.38 (m, 1H, vinylic H); 4.58 (br. s, 1H, H-C(3′)); 4.03 (br, d, 1H, J= 3.3, H-C(4′)); 3.95–3.80 (m, 2H, H-C(5′)); 2.45–2.32 (m, 2H, H-C(2′)); 2.16–0.84 (m, 11H, H of heptene). MALDI-TOF MS; calcd. for 325.70 $[M + H]^+$; found 325.80.

Preparation of 5-heptenyl-2′-deoxy-5′-*O*-(4,4-dimethoxytrityl)-uridine (3b). 5-Heptenyl-2′-deoxyuridine (2b) (600 mg, 1.85 mmol) was dissolved in dry pyridine and coevaporated three times. 4, 4′-dimethoxytrityl chloride (752 mg, 2.22 mmol), *N, N*-dimethylamino pyridine (68 mg, 0.56 mmol) and triethylamine (310 μl, 2.22 mmol) were added to a solution of 2b in dry pyridine (10 ml). The solution was stirred at ambient temperature under a nitrogen atmosphere for 16 h. The reaction mixture was extracted with EtOAc (100 ml x 3) and water (150 ml). The organic layer was collected, dried over anhydrous Mg_2SO_4, filtered, and evaporated to dryness under reduced pressure. The crude product was then purified by silica gel column chromatography ($CHCl_3$/EtOH 97:3) to afford 3b (863 mg, 1.38 mmol, 75%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (br. s, 1H, H-C(6)); 7.45–7.20 (m, 8H, arom.H); 6.83–6.80 (m, 5H, arom. H); 6.39 (t, 1H, J= 6.9, H-C(1′)); 6.27–6.17(m, 1H, vinylic H); 5.57–5.52 (d, 1H, J= 15.9, vinylic H); 4.54 (br, s, 1H, H-C(3′)); 4.05–4.01 (m, 1H, H-C(4′)); 3.78 (s, 6H, H of methoxyl); 3.53–3.31 (m, 2H, H-C(5′)); 2.43–2.18 (m, 2H, H-C(2′)); 1.98–0.73 (m, 11H, H of heptene). MALDI-TOF MS; calcd. for 649.29 $[M + Na]^+$; found 649.29.

Preparation of 5-heptenyl-2′-deoxy-5′-*O*-(4,4-dimethoxytrityl)-uridine phosphoramidite (4b). 5-heptenyl-2′-deoxy-5′-*O*-(4,4-dimethoxytrityl)uridine (3b) (235 mg, 0.38 mmol) in dry CH_3CN (1.5 ml) in a sealed bottle with septum was dissolved in dry acetonitrile and coevaporated three times in *vacuo.* After substitution with nitrogen, 2-cyanoethyl *N, N, N′, N′*-tetraisopropyl phosphorodiamidite (120 μl, 0.38 mmol) in dry acetonitrile (1.5 ml), and 0.5 M tetrazole (0.95 ml) were added, and the reaction mixture was stirred at ambient temperature for 2 h. The reaction mixture was then extracted with AcOEt (10 ml x 3) and water (15 ml). The organic layer was collected, dried over anhydrous Mg_2SO_4, filtered, and evaporated to dryness under reduced pressure. Then, the crude product cyanoethylphosphoramidite of **4b** (316 mg, 0.38 mmol, quant.) in a sealed bottle with septum was dissolved in dry acetonitrile and coevaporated three times, and was used for automated DNA synthesis without further purification.

Synthesis and characterization of ^{H}U-containing ODN

^{V}Uderivatives-containing ODN were synthesized by the automated solid-phase phosphoramidite method as reported. [S1] After automated synthesis, the oligomer was deprotected by incubation with 28% ammonia for 4 h at 65 °C and was purified on a Chemcobond 5-ODS-H column (4.6 × 150 mm) by reverse phase HPLC; elution was with 0.05M ammonium formate containing 3–20% acetonitrile, linear gradient (30 min) at a flow rate of 1.0 mL/min, 30 °C. Preparation of oligonucleotides was confirmed by MALDI-TOF-MS analysis.

MALDI-TOF MS: calcd. 7114.95 for ODN (^{V}U) $[(M + H)^+]$, found 7114.20).

MALDI-TOF MS: calcd. 7185.08 for ODN (^{H}U) $[(M + H)^+]$, found 7184.95).

MALDI-TOF MS: calcd. 7197.09 for ODN(^{HV}U) $[(M + H)^+]$, found 7197.76).

MALDI-TOF MS: calcd. 7171.05 for ODN (^{BuV}U) $[(M + H)^+]$, found 7171.08).

Photochemical detection of 5-methylcytosine on a DNA chip using 5-vinyl-2′-deoxyuridine derivatives

To demonstrate that template-directed photoligation by using photosensitive ODNs could be incorporated into platforms suitable for DNA chip technologies, we constructed the DNA chip by attaching amino-labeled ODN containing ^{V}U derivatives onto the aldehyde-modified glass surface. We determined the feasibility of the template-directed photoligation through photosensitive ODNs on a DNA chip. A glass chip spotted with 2 μM target ODN(C) or ODN(^{m}C) and template ODN was irradiated at 366 nm for 1 h in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride. After the chip had been washed with deionized water at 98 °C for 5 min, 5 μM Cy3-containing ODN(Cy3) conjugate was added to the surface for 4 h in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride, and the chip was washed twice in PBS. Fluorescence signals were detected on a microarray scanner.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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