DNA Recognition

DOI: 10.1002/ange.200600790

SNP Genotyping by Using Photochemical Ligation**

*Shinzi Ogasawara and Kenzo Fujimoto**

A DNA chip for analysis of biomedical features, such as single-nucleotide polymorphisms (SNPs), is a most powerful and useful tool, which allows the simultaneous detection of many different target molecules present in a sample.[1] However, the technology is not yet widely used in routine clinical settings, because it requires improvement in terms of fidelity, reproducibility, and expedition, all of which are essential for standardization and human-gene diagnosis. Whether electrochemical detection[2] or fluorescence detection,[3] the reaction principle that underlies the DNA-chip assays is hybridization with allele-specific oligonucleotide (ASO) probes. In allele-specific hybridization, the difference in thermal stability between perfectly matched and mismatched ASO probes with the DNA target is used to distinguish between SNP alleles. This difference can be very small and can vary based on sequence context. Therefore, although ASO probes can distinguish single-nucleotide differences based on small changes in hybridization efficiency, the

[*] S. Ogasawara, Prof. Dr. K. Fujimoto
School of Materials Science
Japan Advanced Institute of Science and Technology
Asahidai, Nomi, Ishikawa 923-1292 (Japan)
Fax: (+81) 761-51-1671
E-mail: kenzo@jaist.ac.jp
Prof. Dr. K. Fujimoto
PRESTO
Japan Science and Technology Agency (JST)
Kawaguchi 332-0012 (Japan)

[**] This work was supported by Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST), Japan. Partial support by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, is also acknowledged. SNP = single-nucleotide polymorphism.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

selectivity and sensitivity seen for ASO probes of 15 oligodeoxynucleotides or longer is relatively small.

As a result of this problem, there has been considerable recent work to find molecular strategies for increasing selectivity toward such small genetic differences. One of the most common strategies currently used to engender high specificity in sequence detection is the use of DNA-ligation enzymes.[4] Ligases, such as the T4 and Tth enzyme, are quite sensitive to base mismatches at the ligation junction, and selectivity against single mismatches is high, in the order of 10- to 10^2-fold. Although they are quite useful, enzymatic ligation methods for sequence detection also face some limitations. For example, the ligation efficiency on the solid phase is relatively lower than in a solution.[5] In addition, there are the limits in the most suitable conditions, including temperature, pH value, and salt concentration, caused by the use of an enzyme. Indeed, the available range of salt (Mg^{2+}) concentrations for the use of T4 ligase was 10–100 mM, as shown in a separate experiment (see the Supporting Information). DNA ligases also show low activity with RNAs.[6] Finally, ligase-mediated approaches are unlikely to be useful with modified probes that contain non-natural DNA backbones, such as peptide nucleic acids, phosphoramidate DNA, or 2′-*O*-methyl RNA. Even relatively simple modifications, such as conjugation with biotin or fluorescent labels, may be expected to cause difficulties near the ligation junction.

Therefore, as a more useful strategy, the consideration of alternatives to ligase enzymes in the joining of DNA strands seems justified for some applications. A number of nonenzymatic methods that involve the addition of ligating reagents to the DNA have been described.[7] However, even with an autoligation approach, which is considered to be the most useful method for single-mismatch discrimination, the ligation efficiency reached only 80% after 7 hours.[7c] Additional efforts are thus needed to create more broadly applicable methods that would allow accurate, rapid, and selective identification of SNPs. Herein, we describe a novel SNP-typing method which is based on template-directed[8] photochemical ligation of DNA, by using 5-carboxyvinyldeoxyuridine (^{cv}U) as previously reported.[9] The photochemical-ligation approach offers the potential advantages of rapid analysis, sequence selectivity, less-restricted reaction conditions, and high efficiency on RNA targets, thereby rivaling the use of ligase enzymes.[9d] In our method, an unusual salt-concentration-dependent hybridization behavior associated with the probe strand was exploited to achieve selectivity without an annoying requirement for thermal stringency during the photochemical-ligation reaction.

Figure 1A shows a schematic representation of the strategies employed. We chose a strand design that involved the ligation of a 9-mer strand (capture) containing ^{cv}U at the 5′-end and a biotin-tagged 21-mer strand (probe), which can afford very high sequence specificity because of the high mismatch selectivity of the shorter capture strand. The capture and probe strands were complementary to a sequence encompassing amino acid residue 248, called a hot spot, on exon 7 of the human oncogene p53,[10] which has been found to be mutated in more than 50% of known human cancers.

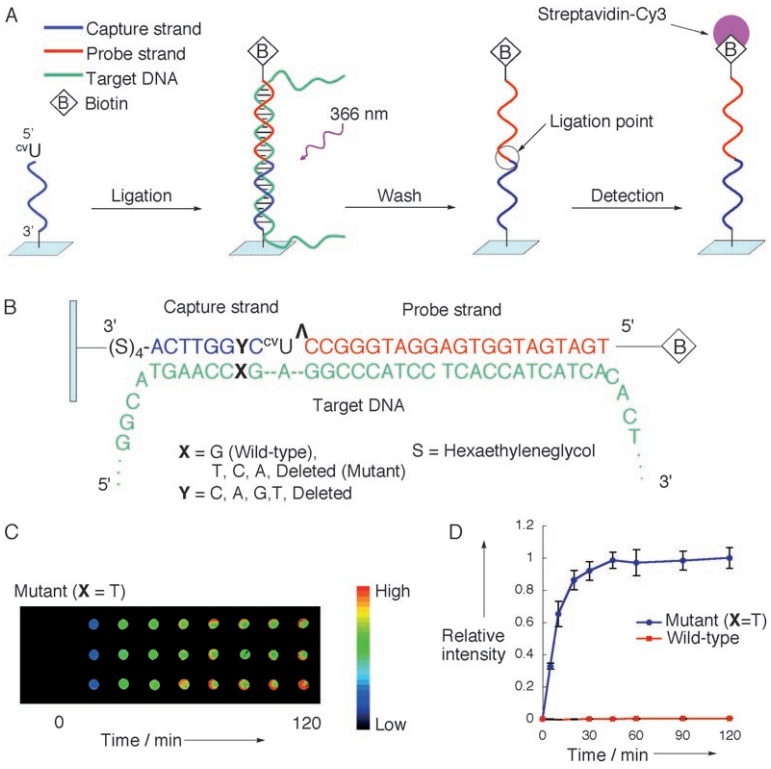

Figure 1. Scheme, sequence, and progress of the photochemical ligation. A) Conceptual scheme showing how the target is detected by photochemical-ligation and hybridization specificity. B) Capture, probe, and target sequences used in this study. C) Fluorescence image for a time course of mutant detection. The capture strands (**Y** = A) were attached to all 27 spots, and three longitudinal spots were irradiated at 366 nm during the same period. D) Differing rates of photochemical ligation of matched and singly mismatched target DNA strands with the mutant-detection capture strand (**Y** = A).

To investigate the difference in the ligation rate between the matched and mismatched sequences on the DNA chip, we prepared two capture strands (mutation and wild type) and target strands with the p53 mutant sequence (Figure 1B). Capture strands were immobilized onto the activated glass surface by spotting with solutions of the appropriate 3′-amino-modified capture strands in sodium cacodylate buffer (see the Experimental Section and Supporting Information). Ligations were carried out on the DNA chip by 366-nm irradiation at room temperature with the 5′-biotin-tagged probe and the target, in a tris(hydroxymethyl)aminomethane–HCl (Tris-HCl) buffer containing Mg^{2+} ions. After the chip was washed twice with phosphate-buffered-saline (PBS) solution and once with ultrapure water at 98 °C for 10 minutes, streptavidin-Cy3 conjugate was added. Surface fluorescence measurements were performed on a CRBIO IIe microscanner array (Hitachi Software Engineering Co.) after washing twice with a PBS solution at room temperature. The results show that the reaction takes place on the target over a period of hours and reaches the 50% stage within 10 minutes (Figure 1C,D). Significantly, a single mismatch in the target sequence yielded very little ligated product, with a measured rate that was 10^3-fold lower than that of the complementary case.

In such a ligation reaction, there are two possible sources of specificity: the ligation reaction itself and the hybridization selectivity between the target and capture strands. We also carried out mutation detection in the conventional way for the same target to verify the potential selectivity of our photochemical ligation for SNP detection. A conventional way of increasing target selectivity is to wash the DNA chip with a buffer solution at an adequate temperature, which results in the dehybridization of DNA duplexes formed from noncomplementary strands. In the mutation detection by the traditional method, which shows only hybridization specificity, the observed selectivity for the matched (mutant) sequence was about 8, even after washing with a stringent solution at the appropriate temperature (Figure 2A). One possible reason for the decrease in selectivity is the loss of DNA duplexes of matched sequences, particularly during the washing step employed to remove noncomplementary strands from the DNA chip. A second possible source of low selectivity is incomplete washing of the mismatched sequences (wild type). By contrast, the fluorescence image after use of the photochemical-ligation method showed no loss of the biotin-tagged probe strand, due to covalent bonding between the capture and probe strands (Figure 2B). Moreover, mismatched duplexes and other components with the potential to harm the fluorescence imaging were eliminated completely by high temperatures (Figure 2C,D). The influence of the washing conditions is summarized in Figure 2E.

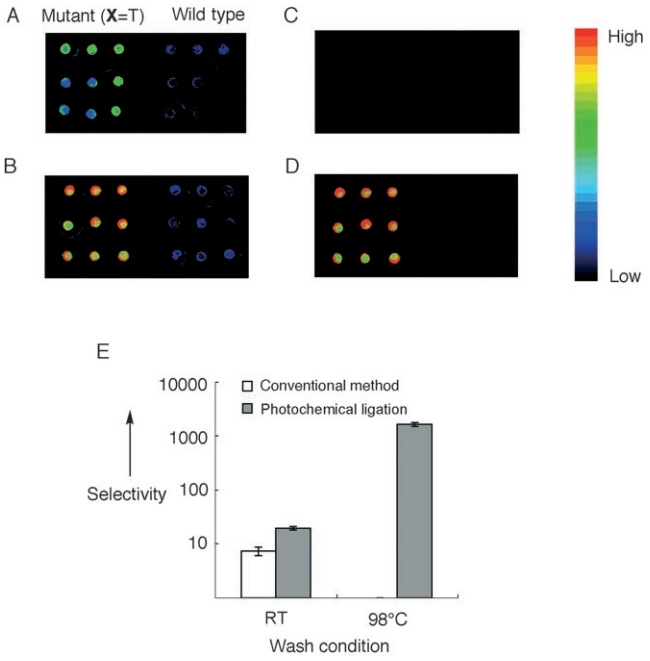

Figure 2. Influence of washing and photochemical ligation on specificity. In all four fluorescence images, the capture strands (**Y** = A) were attached to the surface, and the targets (left: **X** = T, 3×3 spots; right: **X** = G, 3×3 spots) were applied. A) Use of only hybridization specificity and washing with buffer under stringent conditions (room temperature). B) Use of both hybridization specificity and photochemical-ligation specificity and washing with buffer under stringent conditions. C) Use of only hybridization specificity and washing with ultrapure water at 98 °C for 10 min. No signal was observed. D) Use of both hybridization specificity and photochemical-ligation specificity and washing with ultrapure water at 98 °C for 10 min. E) Selectivity for different washing conditions.

Finally, to demonstrate the generality of sequence discrimination, we constructed a set of five closely related targets (all in the p53 context) with a single variable base (A, G, C, T, or deleted (D)), along with 9-mer immobilized capture strands with the same five bases. We then carried out kinetic measurements for all 25 possible combinations of capture and target strands (Table 1). The ligation of each of the 20 possible mismatched sequences is presented as a ratio between the ligation efficiency of each mismatched reaction and that of the corresponding matched one. Most mismatches are discriminated by a factor greater than 10^3-fold, as compared to the corresponding matched sequence (G–A, G–D, G–G, C–T, C–D, C–C, A–C, A–G, A–D, A–A, T–C, D–T, D–C), and almost all of the remaining sequences are discriminated by greater than 10^2-fold (Figure 3).

Table 1: Survey of photochemical-ligation rates for all base-pairing combinations in the context of the p53 system.

Base pair (**X**–**Y**)[a]	Relative intensity[b]	Base pair (**X**–**Y**)[a]	Relative intensity[b]
G–C	1.0 ± 0.18	T–D	0.0029 ± 0.0039
G–T	0.013 ± 0.008	T–T	0.016 ± 0.001
G–A	0[c]	A–T	1.0 ± 0.15
G–D	0[c]	A–C	0[c]
G–G	0[c]	A–G	0[c]
C–G	1.0 ± 0.18	A–D	0[c]
C–T	0[c]	A–A	0[c]
C–A	0.012 ± 0.008	D–D	1.0 ± 0.13
C–D	0.0009 ± 0.0021	D–T	0[c]
C–C	0[c]	D–C	0.0010 ± 0.0089
T–A	1.0 ± 0.16	D–A	0.0031 ± 0.0022
T–C	0.0005 ± 0.0013	D–G	0.0021 ± 0.0051
T–G	0.018 ± 0.014		

[a] D stands for nucleic base **X** or **Y** deleted. [b] Intensity of the mismatched-ligation signal divided by that of the corresponding correctly matched-ligation signal; three data points were used in each case. [c] The average intensity as measured is approximately equal to zero.

In summary, we have developed a new SNP-typing method with high selectivity and sensitivity by introducing a form of photochemical-ligation technology into an existing allele-specific hybridization. Washing of a DNA chip after photoirradiation without thermal stringency and combination of photochemical-ligation specificity with hybridization specificity between the target and capture strands is shown to produce discrimination of point mutations up to 10^3-fold and more. In principle, one might use other types of photosensitive molecules, such as *p*-carbamoylvinyl phenol nucleosides,[11] to catch target molecules directly without the extra probe strand. This new protocol should facilitate the expeditious, reliable, and parallel screening of important SNPs and could be readily scaled up for a high-throughput automated operation.

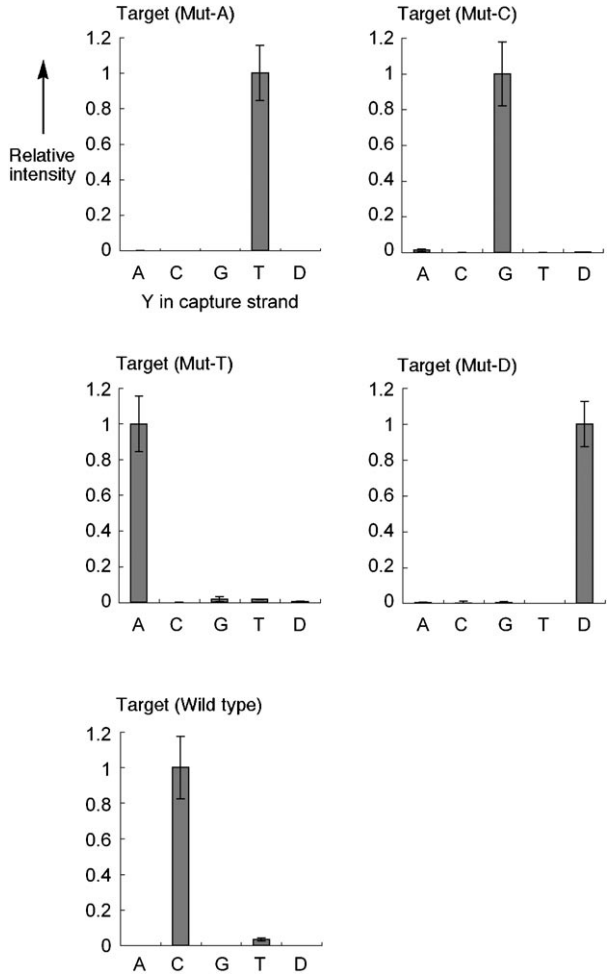

Figure 3. Relative intensity for all 25 possible combinations of capture and target strands. Each experiment was repeated at least three times.

Experimental Section

^{cv}U was synthesized from 5-iodo-2′-deoxyuridine; the method was reported previously.[8] ^{cv}U-containing capture strands were prepared by the β-cyanoethylphosphoramidite method on a 3′-amino-modified C7 CPG 500 resin (purchased from Glen Research) by using an Applied Biosystems 3400 DNA synthesizer and the standard method. After automated synthesis, the oligomers were cleaved and deprotected with concentrated aqueous ammonia at 55 °C for 17 h, and purified by HPLC. The capture strands were characterized by MALDI-TOF MS.

The amino-modified capture strands were diluted to a concentration of 30 μM in 100 mM sodium cacodylate buffer (pH 7.4) and 100 mM NaCl. DNA solution (1 μL) was dispensed onto the aldehyde glass slide (purchased from Nalge Nunc International Co.) and incubated for 12 h in a desiccator. After incubation, the glass slides were washed twice with 0.15% sodium dodecylsulfate (SDS) and twice with ultrapure water and were then incubated for 5 min with blocking solution (10 mg $NaBH_4$ dissolved in PBS (4 mL) and 100% ethanol (1 mL)). After the blocking, the slide was dipped 20 times into ultrapure water and then dried.

The photochemical-ligation reaction was performed by exposure of the surface to 1 nM target (5 attomoles), 1 μM 5′-biotin-labeled probe strand in Tris-HCl buffer (pH 7.6), and 250 mM $MgCl_2$. A 5-μL drop of this solution was placed onto the DNA chip and then spread over the entire surface by placing a clean quartz cover glass on top of the sample, after which the DNA chip was photoirradiated at 366 nm with transilluminator. The photochemical-ligation reaction was allowed to proceed for 60 min at room temperature, after which the surface was rinsed twice with 0.15% SDS and twice with ultrapure water at 98 °C for 10 min.

Received: March 1, 2006
Published online: May 31, 2006

Keywords: biosensors · DNA recognition · ligation reactions · medicinal chemistry · photochemistry

[1] a) A. Marshall, J. Hodgson, *Nat. Biotechnol.* **1998**, *16*, 27–31; b) G. Ramsay, *Nat. Biotechnol.* **1998**, *16*, 40–44.
[2] a) G. C. King, D. A. Giusto, W. A. Wlassoff, S. Giesebrecht, E. Flening, G. D. Tyrelle, *Hum. Mutat.* **2004**, *23*, 420–425; b) J. Wang, G. O. Liu, A. Merkoci, *J. Am. Chem. Soc.* **2003**, *125*, 3214–3215; c) F. Patolsky, E. Katz, I. Willner, *Angew. Chem.* **2002**, *114*, 3548–3552; *Angew. Chem. Int. Ed.* **2002**, *41*, 3398–3402; d) J. Park, T. A. Taton, C. A. Mirkin, *Science* **2002**, *295*, 1503–1505; e) E. M. Boon, D. M. Ceres, T. G. Drummond, M. G. Hill, J. K. Barton, *Nat. Biotechnol.* **2000**, *18*, 1096–1100.
[3] a) H. Matsuzaki, S. Dong, H. Loi, X. Di, G. Liu, E. Hubbell, J. Law, T. Berntsen, M. Chadha, H. Hui, G. Yang, G. Kennedy, T. A. Webster, S. Cawley, P. S. Walsh, K. W. Jones, S. P. A. Fodor, R. Mei, *Nat. Methods* **2004**, *1*, 109–111; b) D. A. Hinds, L. L. Sture, G. B. Nilson, E. Halperin, E. Eskin, D. G. Ballinger, K. A. Frazer, D. R. Cox, *Science* **2005**, *307*, 1072–1079; c) C. Y. Zhang, H. C. Yen, M. T. Kuroki, T. H. Wang, *Nat. Mater.* **2005**, *4*, 826–831.
[4] a) H. Baron, S. Fung, A. Aydin, S. Bahring, F. C. Luft, H. Schuster, *Nat. Biotechnol.* **1996**, *14*, 1279–1282; b) N. P. Gerry, N. E. Witowski, J. P. Day, R. P. Hammer, G. Barany, F. Barany, *J. Mol. Biol.* **1999**, *292*, 251–262; c) R. Favis, J. P. Day, N. P. Gerry, C. Phelan, S. Narod, F. Barany, *Nat. Biotechnol.* **2000**, *18*, 561–564.
[5] A. G. Frutos, L. M. Smith, R. M. Corn, *J. Am. Chem. Soc.* **1998**, *120*, 10277–10282.
[6] M. Nilson, G. Barbany, D. Antson, K. Gerton, U. Landegren, *Nat. Biotechnol.* **2000**, *18*, 791–793.
[7] a) Z. Y. Zhan, D. G. Lynn, *J. Am. Chem. Soc.* **1997**, *119*, 12420–12421; b) P. Lou, J. C. Leitzel, Z. Y. Zhan, D. G. Lynn, *J. Am. Chem. Soc.* **1998**, *120*, 3019–3031; c) S. Sando, E. T. Kool, *J. Am. Chem. Soc.* **2002**, *124*, 2096–2097.
[8] a) M. M. Rozeman, D. R. Liu, *ChemBioChem* **2006**, *7*, 253–256; b) X. Li, D. R. Liu, *Angew. Chem.* **2004**, *116*, 4956–4979; *Angew. Chem. Int. Ed.* **2004**, *43*, 4848–4870.
[9] a) K. Fujimoto, S. Matsuda, N. Takahashi, I. Saito, *J. Am. Chem. Soc.* **2000**, *122*, 5646–5647; b) S. Ogasawara, K. Fujimoto, *ChemBioChem* **2005**, *6*, 1756–1760; c) M. Ogino, Y. Yoshimura, A. Nakazawa, I. Saito, K. Fujimoto, *Org. Lett.* **2005**, *7*, 2853–2856; d) Y. Yoshimura, Y. Noguchi, H. Sato, K. Fujimoto, *Nucleic Acid Symp. Ser.* **2005**, *49*, 143–144.
[10] P. Hainaut, T. Hernandez, A. Robinson, P. Rodriguez-Tome, T. Flores, M. Hollstein, C. C. Harris, R. Montesano, *Nucleic Acids Res.* **1998**, *26*, 205–213.
[11] Y. Yoshimura, Y. Ito, K. Fujimoto, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1295–1298.

