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An effective method for the in situ synthesis of DNA–CPG conjugates using chemical ligation technology as tools for SNP analysis

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Abstract—In this paper, we report a new method for the SNP analysis by using a chemical ligation (CL) technique on CPG plates with high coupling efficiency. This method showed markedly high match/mismatch discrimination ability. Particularly, replacement of thymidine with 2-thiothymidine in DNA probes used in the CL technology resulted in significant improvement of the base discrimination ability of the thymine base in this system.

DNA and RNA molecules can recognize the complementary oligonucleotides by use of Watson–Crick base pairs of A–T and G–C for the maintenance of life. In the previous study, a number of methods have been developed for the suppression of specific genes,[1,2] the exhaustive analysis of gene expression,[3] and the detection of single nucleotide polymorphisms (SNPs)[4] by using the base recognition. Among them, the SNPs detection plays an important role in identification of disease-causing genes and administration of suitable drugs.[5]

The SNPs detection for rapid simultaneous analysis of many samples is generally carried out by using DNA chips consisting of oligodeoxynucleotide probes immobilized on the surface of supports.[6] However, the previous DNA chip strategy has serious problems of reproducibility and fidelity. One of their reasons might be low purity of DNA probes on slide glasses. The efficiency of the reactions (coupling, capping, oxidation, and deprotection) required for each chain elongation on slide glasses was markedly lower[7] than that on controlled pore glasses (CPG)[8] or highly cross-linked polystyrene polymer supports[9] which are used for the current synthesis of DNA fragments. If the average of the coupling efficiency is ca. 95% in the *on-chip* synthesis of a DNA probe 25mer, the purity of the desired oligonucleotide probe is estimated to be less than 30%. Therefore, ca. 70% of all probes in such a case might have incorrect sequences. This low purity of DNA probes resulted in decrease of selectivity and sensitivity of DNA chips. To avoid this problem, Tsukahara and Nagasawa have recently developed a new strategy called 'probe-on-carrier method' for SNPs detection.[10] In their method, CPG was used as a support containing DNA probes instead of a slide glass.

On the other hand, Kool and co-workers reported that the SNP analysis based on the chemical ligation using DNA probes having a 3′-thiophosphate group on polystyrene beads showed high detection ability.[11] In this paper, we propose a new strategy for the in situ synthesis of DNA–CPG conjugates having a 5′-terminal iodo group to improve the CL technology for its application to SNP analysis, as shown in Figure 1.

Keywords: Chemical ligation; Gene detection; SNP; N-unprotected DNA synthesis; 2-Thiothymidine; Base recognition.
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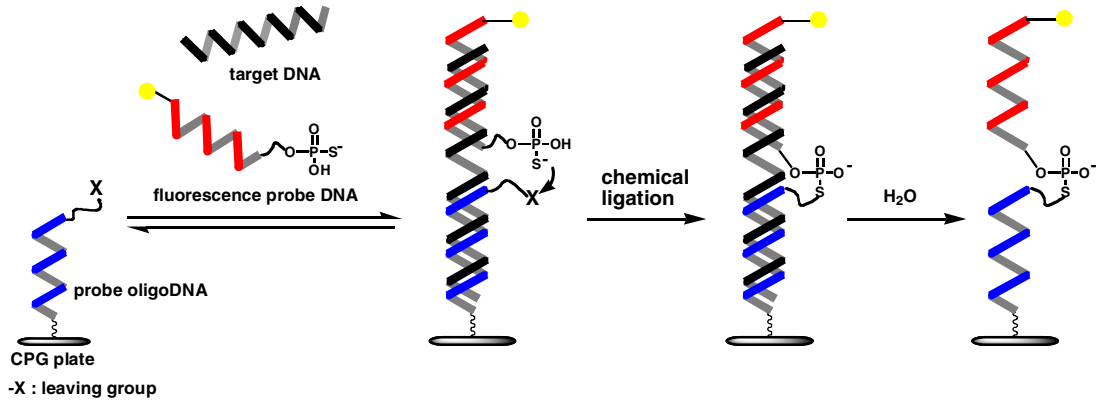


Figure 1. SNP analysis using a chemical ligation technology on CPG plates.

To see if an oligoDNA having an iodide residue in the DNA synthesis is stable, 5′-iodo-5′-deoxythymidyl(5′-3′)thymidine (**2**: ^{I}TpT) was synthesized as a model compound, as shown in Figure 2. The protected dimer **1** was synthesized on CPG in the general phosphoramidite method. Although the protected dimer **1** was seriously decomposed when treated with 2 M $MeNH_2$/THF for 1 h[12] or DBU–BSA[13] even for 10 min, it was found that the protected species **1** was converted to the desired compound **2** in 72% isolated yield by treatment with concd NH_3 at room temperature for 1 h. This result suggested the iodo group was stable under these conditions. However, we have noticed a serious problem that most of the DNA probes synthesized were eliminated from CPG when treated with concd NH_3 for longer periods of time required for removal of the base protecting groups.[7,14] Since it is necessary to preserve oligoDNA probes on the plate for the CL strategy, the deprotection step using concd NH_3 should be remarkably shortened. Therefore, we chose the activated phosphite method without base protection by use of N-unprotected deoxynucleoside 3′-phosphoramidite building blocks. After the chain elongation cycle was over, the probe-CPG plate was treated with concd NH_3 at room temperature for shorter periods of time (5 min) to selectively remove the 2-cyanoethyl groups without elimination of the probe. The deprotection of the 2-cyanoethyl group by treatment with concd NH_3 for 5 min on CPG was confirmed by HPLC analysis (data not shown).

Next, we carried out several model experiments of SNPs analysis by using non-fluorescence-labeled samples **5** of a P450 gene,[15] fluorescence-labeled DNA probe **4**, and DNA probe **3** synthesized on uniformly flattened square plate of CPG (probe-CPG plate) with thickness of 1 mm. As shown in Figure 3, the match/mismatch discrimination for the P450 SNP site sequences (wild type; XY = TA (**5a**), 1075 mutant; GA (**5b**), and 1076 mutant; TG (**5c**)) was analyzed by use of the probe-CPG plate **3** synthesized in the activated phosphite method along with three kinds of fluorescence-labeled DNA probes (wild type; X′Y′ = AT (**4a**), 1075 mutant; CT (**4b**), and 1076 mutant; AC (**4c**)). To examine the purity of DNA probe **3** in the CPG plate, we also carried out

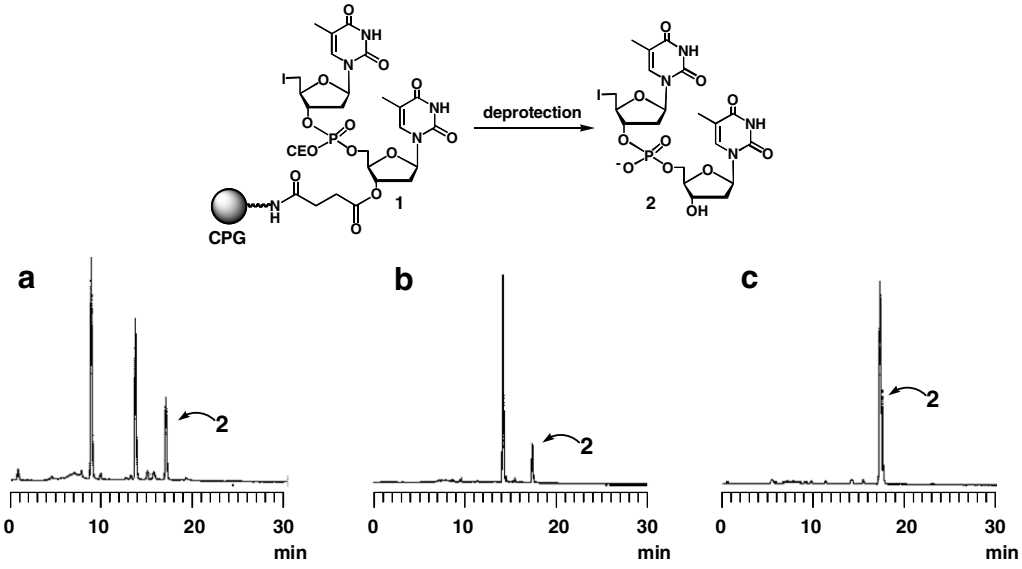
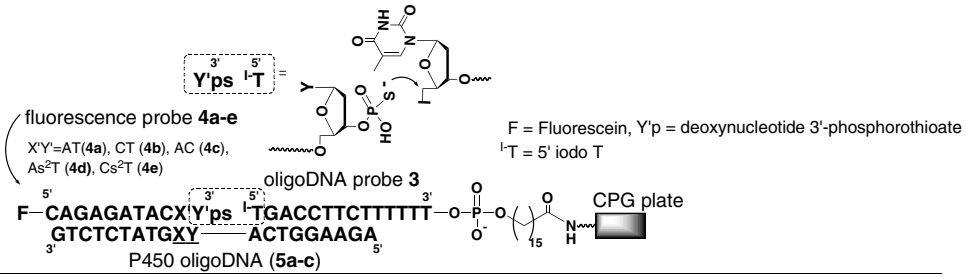


Figure 2. The reverse-phase HPLC profiles of the crude mixtures obtained in the synthesis of **2** having a 5′-iodo-5′-deoxythymidine under different conditions. (a) Deprotection by treatment with 2 M $MeNH_2$/THF for 30 min; (b) 10% DBU in pyridine–BSA (1:1, v/v) for 10 min, then, concd NH_3 for 1 h; (c) concd NH_3 for 1 h.



	DNA 20mer **5a** having a wild type sequence of P450 XY = TA		DNA 20mer **5b** having a 1075-mutant sequence of P450 X Y = GA		DNA 20mer **5c** having a 1076-mutant sequence of P450 XY = TG	
	match or mismatch	ratio of fluorescence strength	match or mismatch	ratio of fluorescence strength	match or mismatch	ratio of fluorescence strength
wild-type probe **3**, **4a** (X'Y' = AT)	match	1	G - A	0.11	G - T	0.39
1075-mutantprobe **3**, **4b** (X'Y' = CT)	T - C	0.18	match	1	G - T, T - C	0.27
1076-mutant probe **3**, **4c** (X'Y' = AC)	A - C	0.17	G - A, A - C	0.09	match	1
wild-type probe **3**, **4d** (X'Y' = As2T)					G - s2T	0.059
1075-mutantprobe **3**, **4e** (X'Y' = Cs2T)					G - s2T, T - C	0.035

Figure 3. Match/mismatch discrimination of the 20mer-DNAs having sequences of P450 in the chemical ligation system by using an oligoDNA probe having a iodo thymidine at the 5′ position and a fluorescence-labeled probe DNA having a thiophosphate group at the 3′ position on CPG plates.

the DNA synthesis using CPG resins containing a succinyl linker in the activated phosphite method. After the chain elongation was finished, the resulting oligonucleotide was eliminated from the resin by treatment with concd NH_3 at room temperature for 1 h. The HPLC analysis of the mixture thus obtained showed an almost single peak of the desired oligomer (Fig. 4), which, *in turn*, was characterized by MALDI-TOF mass spectroscopy. These results indicated that the purity of the DNA–CPG conjugate synthesized in the activated posphite method was very high.

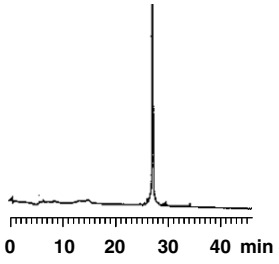
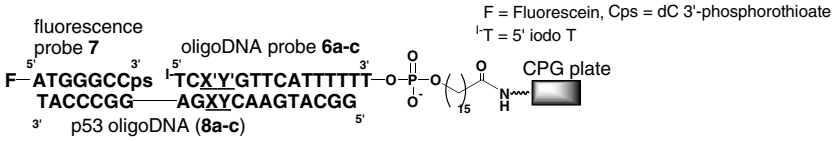


Figure 4. The anion-exchange HPLC profile of the crude mixtures obtained in the synthesis of I-TGACCTTCTT$_5$ by using activated phosphite.

When XY was TA, there was more than fivefold discrimination ability of the matched sequence toward the T–C (=X–X′) or A–C (=Y–Y′) mismatches.[16] When XY was GA, there was a ca. 10-fold difference in discrimination between the matched sequence and the one or two mismatched sequences. However, when XY was TG, the discrimination between the matched and mismatched sequences was very low (by only 2.5-fold strength of fluorescence). The same tendency was observed in the p53 SNPs analysis by using a probe having a recognition site on porous glass, as shown in Figure 5. The match/mismatch discrimination of the p53 SNP site



	DNA 20mer **8a** having a wild type sequence of p53 XY = GC		DNA 20mer **8b** having a HSC-4 sequence of p53 X Y = AC		DNA 20mer **8c** having a Ca9-22 sequence of p53 XY = GT	
	match or mismatch	ratio of fluorescence strength	match or mismatch	ratio of fluorescence strength	match or mismatch	ratio of fluorescence strength
wild-type probe **6a**, **7** (X'Y' = CG)	match	1	A - C	0.11	T - G	0.36
HSC4 mutant probe **6b**, **7**(X'Y' = TG)	G - T	0.44	match	1	G - T, T - G	0.18
Ca9-22 mutant probe **6c**, **7** (X'Y' = CA)	C - A	0.19	C - A, A - C	0.11	match	1

Figure 5. Match/mismatch discrimination of the 20mer-DNAs having sequences of p53 in chemical ligation system by using an oligoDNA probe having a iodo thymidine at the 5′ position and a fluorescence-labeled probe DNA having a thiophosphate group at the 3′ position on CPG plates.

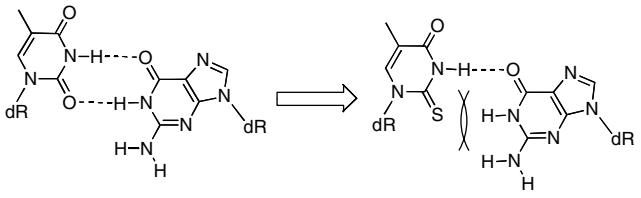


Figure 6. Structures of G–T and G–s^2T wobble base pairs.

sequences (wild type; XY = GC (**8a**), HSC-4 mutant; AC (**8b**), and Ca9-22 mutant; GT (**8c**)) along with wild-type probe; X′Y′ = CG (**6a**), HSC-4 mutant probe; X′Y′ = TG (**6b**), and Ca9-22 mutant probe; X′Y′ = CA (**6c**)[17] was analyzed by using three kinds of probe-CPG plates (wild type; DNA probe **7**). There was a ca. five-fold difference in discrimination of the matched sequence (**8a**–**6a**) toward the C–A mismatch (**8a**–**6c**). Moreover, a ca. ninefold difference in the discrimination ability of the matched sequence (**8b**–**6b**) toward the C–A mismatch (**8b**–**6a**) was observed, though only a 1.9-fold recognition ability was observed when a 20mer DNA probe was used without the CL (data not shown).[18] This high discrimination ability in the CL strategy results from the difference in conditions of washing because the solvent for washing in the CL strategy was H_2O instead of the sodium phosphate buffer containing NaCl. However, there were only 2.2- to 2.8-fold discrimination abilities of the matched sequence (**8a**–**6a**, **8c**–**6c**) toward the T–G mismatch (**8a**–**6b**, **8c**–**6a**).

Since oligonucleotides containing a 2-thiothymidine (s^2T) derivative can destabilize a wobble base pair with the opposite guanine,[19] as shown in Figure 6, s^2T was introduced into the SNP recognition site (X′Y′ = As^2T (**4d**) and Cs^2T (**4e**)) to increase the discrimination ability between the G–C and G–T base pairs. The discrimination between G–C and G–s^2T on the CPG plate was dramatically improved to be 17-fold higher than that between G–C and G–T, as shown in Figure 3. These results showed that this method could be easily applied to the use of DNA chips in situ synthesized on CPG plates.

In summary, we first demonstrated the utility of DNA–CPG conjugates showing the successful SNPs detection in combination with the CL strategy. Particularly, the activated phosphite method recently developed by us could provide DNA–CPG conjugates having a 5′-terminal 5′-iodo-5′-deoxythymidine residue which was a key material in the CL strategy and could not be synthesized by the conventional procedure used for the current DNA synthesis. The DNA–CPG conjugate systems used for SNPs analysis showed markedly high match/mismatch discrimination ability. In particular, the use of 2-thiothymidine in place of thymidine proved to be of great importance for enhancement of the base discrimination ability of the thymine base in this system. The present method would be useful for the essential improvement of the sensitivity and selectivity of gene expression analysis and SNPs detection. Further studies are now under way in this direction.

Acknowledgments

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16. The CPG plate was added to a solution of a target oligoDNA (1.60 μM) and a fluorescence probe (1.60 μM) in 100 mM sodium phosphate buffer (1 M NaCl, 50 μM DTT, pH 7.0). The mixture was incubated at 40 °C for 14 h. After that, washing of the glass plate was carried out by using water. After drying the glass plate, the fluorescence strength of the plate was measured by fluorescence microscopy.
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18. The CPG plate was added to a 1.60 μM solution of a fluorescence-labeled target oligoDNA in 100 mM sodium phosphate buffer (1 M NaCl, pH 7.0). The mixture was incubated at 40 °C for 14 h. After that, washing of the glass plate was carried out by using 100 mM sodium phosphate buffer (0.1 M NaCl, pH 7.0). The mixture was incubated at 50 °C for 1 h. After drying the glass plate, the fluorescence strength of the plate was measured by fluorescence microscopy.
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