# Highly sensitive detection of DNA using enzyme-linked DNA-probe. 1. Colorimetric and fluorometric detection

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## ABSTRACT

In order to develop non-radioactive oligonucleotide derivatives and to examine their utility as a diagnostic tool, namely as DNA-probe, an enzyme-linked oligonucleotide was synthesized. Oligonucleotide complementary to M13mp8 phage DNA was linked to alkaline phosphatase via a crosslinker and a spacer. M13mp8 phage DNA (single strand) immobilized on the nitrocellulose membrane was hybridized with the enzyme-linked oligonucleotide. The hybrid was detected with three detection methods;(1)colorimetric detection in solution, (2)colorimetric one on membranes, and (3)fluorometric one in solution. Methods(2) and (3) gave high sensitivities to detect as low as several to several tens attomoles of DNA and it was found that those methods with enzyme-linked oligonucleotides are potent for DNA-probe methodology from the viewpoint of automation.

## INTRODUCTION

DNA-probe methodology becomes popular for diagnosis of diseases by detecting specific DNA sequences. The key requirement for this methodology is to develop non-radioactive and highly sensitive detection system, because the radioactives which have been used by now cause some complicated problems. The non-radioactive detection system must be composed of non-radioactive enhancing steps to accumulate informations from DNA-probes after hybridization. To fulfill the requirements, enzymes, which keep accumulating detectable materials from substrates,have been utilized.

There are two ways to use enzymes for those purposes. One way is to link enzymes with proteins which are highly specific to small molecules. The avidin-biotin complex and the antigen-antibody conjugate are the typical examples(1−4). Biotin and antigens anchored to DNA fragments are detected with enzyme modified proteins. Another is to link enzymes directly to DNA's or oligonucleotides, and the conjugate is used as a DNA-probe(5)(6).

Here we will report a direct method of synthesis of enzyme-linked oligonucleotides using a hetero-bifunctional cross-linking reagent(7), and their ability to detect target DNA fixed on the nitrocellulose membrane. From the viewpoint of automation of DNA-probe diagnosis, three different monitoring methods were examined and their utility was discussed. They are the colorimetic detection in solution, and colorimetric one on membrane and fluorometric one in solution.

## EXPERIMENTAL

### *Materials*

Oligodeoxyribonucleotide, dGTAAAACGACGGCCAGT(P17), which is complementary to M13mp8 phage DNA, was synthesized by Shimadzu Automatic DNA synthesizer (NS-1) with the phosphotriester method and purified by HPLC equipped with a reversed phase

column (Cosmosil C18, 4.6×150mm) using the condition described later. M13mp8 phage DNA (single strand, 100μg/ml, M13-DNA), alkaline phosphatase from calf intestine(CAP), T4-polynucleotide kinase (PNK) were purchased from Nippon Gene Co. Ltd., Boehringer Mannheim Gmbh, and TOYOBO Co. Ltd., respectively. Nitrocellulose membrane (BA85) was purchased from Schleicher & Scheull. N-succinimidyl-3-(2-pyridyldithio)-propionate(SPDP), 4-methylumbelliferyl phosphate (MUP), and 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDCI) were obtained from Pierce Chemical Company Ltd., Sigma Chemical Company, and Nacalai Tesque, respectively. Poly(vinyl alcohol),(PVA), poly(vinylpyrrolidone), (PVP), and poly(ethylene glycol),(PEG) were purchased from Nacalai Tesque.

*Analytical conditions*

A high performance liquid chromatography(HPLC) used was Shimadzu LC-6A equipped with a UV-flow monitor(SPD-6AV). The chromatography conditions were as follows; eluent, A: 5% $CH_3CN$ containing 0.1M triethylammonium acetate(TEAA, pH7) and B: 50% $CH_3CN$ containing 0.1M TEAA; gradient, linear from A to B for 20min ; flow rate,1.0ml; detection,254nm. Gel electrophoresis (PAGE) was carried out with 15% polyacrylamide-7M urea gel in TBE buffer at 1000V for 40min. After electrophoresis, the gel was visualized with a UV shadowing technique.

*Preparation of cystamine linked P17*

P17(30nmol) was phosphorylated with PNK (30units) in Tris-HCl buffer (pH7.6, 30μl). After 1h, the reaction solution was passed through SEP PAK cartridge (C18 type, Waters-Millipore Ltd.) was washed with 25mM triethylammonium carbonate buffer (pH7.5, TEAB) (10ml×2) and 5%$CH_3CN$ in 100mM TEAB(3ml×1). Elution of the oligomer was carried out with 30%$CH_3CN$ in 100mM TEAB (3ml×1) and the eluent was evaporated under reduced pressure until TEAB disappeared. The residue was treated with cystamine dichloride (1M in water, 2μl) and EDCI (1M in water,10μl) in 0.75M 2,6-lutidine-HCl buffer (pH7.5, 88μl). The solution was allowed to react at 20°C. After 18h, the reaction solution was applied to HPLC and the fractions containing cystamine adduct were collected followed by lyophilization (45% in yield). A small part of the residue was treated with 80% AcOH at 30°C for 3h and analyzed by HPLC. The reaction products were also analyzed by PAGE.

*Modification of enzyme with hetero-bifunctional cross-linking reagent, SPDP*

Alkaline phosphatase (CAP, 13nmol) was passed through PD-10 column (Sephadex G-25 prepacked, swelled with 40mM phosphate-buffered saline, PBS) and the first fraction (3ml) was collected. The fraction was condensed to 150μl under reduced pressure and the residual solution (0.8M PBS after condensation) was treated with SPDP (20mM in EtOH, 16μl) for 1h at room temperature with gentle stirring. The reaction solution was passed through PD-10 column and the first fraction (3ml) was collected.

*Estimation of enzyme modification with SPDP.*

A small part of the SPDP-modified CAP solution was treated with DTT(100mM in water,1ml). The concentration of 2-mercaptopyridine released from SPDP was determined by measuring the absorbance at 343nm (molar extinction coefficient: 8080 at 343nm)(8).

*Cross-linking between alkaline phosphatase and oligonucleotide*

Cystamine modified P17 (4nmol) was dissolved in PBS (200mM) and treated with DTT (100mM in water, 100μl) for 4h at room temperature with gentle stirring. The reaction solution was applied to preparative HPLC and the fraction which was newly appeared by the DTT treatment was collected (2.4nmol). The fraction was subjected to cross-linking reaction immediately after evaporation of the fraction to dryness. To the residue, the fraction

containing SPDP-modified CAP (1.2nmol, 0.5ml) was poured, mixed well, and condensed to ca.100$\mu$l under reduced pressure at 4°C. The reaction solution was allowed to react at room temperature with gentle stirring. After 15h, the solution was applied to Bio-Gel P-100 column swelled with PBS(40mM, 10(id)×150mm). The column was eluted with the PBS buffer (40mM) at the flow rate of 0.5ml/min. The molar ratio of CAP against P17 in the adduct was calculated according to equation (1) using the UV spectrum of the adduct and molar extinction coefficients as follows; SPDP-modified CAP: $1.98\times10^5$ at 280nm;$1.06\times10^4$ at 260nm, P17: $8.7\times10^4$ at 280nm; $1.7\times10^5$ at 260nm.

$$\frac{A(\lambda_1)}{A(\lambda_2)} = \frac{X\ \epsilon_{enz}(\lambda_1) + Y\ \epsilon_{oligo}(\lambda_1)}{X\ \epsilon_{enz}(\lambda_2) + Y\ \epsilon_{oligo}(\lambda_2)} \quad (1)$$

where X = molar fraction of the enzyme in the adduct
Y = molar fraction of the oligonucleotide in the adduct
$A(\lambda)$ = absorbance of the adducts at $\lambda$nm
$\epsilon_{enz}(\lambda)$ = molar extinction coefficient of the enzyme at $\lambda$nm
$\epsilon_{oligo}(\lambda)$ = molar extinction coefficient of the oligonucleotide at $\lambda$nm.

*Detection of M13mp8 phage DNA on the nitrocellulose membrane*
M13mp8 phage DNA (single strand, 100$\mu$g/ml) was dissolved in the Tris-HCl buffer (10mM, pH7.5) containing 1mM EDTA to make up solutions with the designated concentration. Aliquots(1$\mu$l) of the solutions were placed on the nitrocellulose membrane. Heat-denaturated salmon sperm DNA(100ng) was placed on the same membrane as a control. After air-drying, the membrane was baked in a vacuum oven at 80°C for 2h and was treated with 1% PVA and 5% PVP for 30min at 40°C. The membrane was pre-hybridized in the hybridization buffer (80mM Tris-HCl buffer(pH 7.8) containing 0.6M NaCl and 4mM EDTA, 6%PEG, 2×Denhaldt's solution, 0.1%SDS) at 40°C for 30min. Hybridization was carried out in the hybridization buffer containing CAP-P17 (10pmol of P17/ml) at 40°C for 1h. The membrane was rinsed with sodium chloride(150mM)-sodium citrate(15mM) buffer(SSC) containing 0.2% SDS at 20°C for 5min, 40°C for 5min, and 20°C for 5min, sequentially(9). The membrane was treated with following procedures.
*Method(1) Colorimetric detection in solution.*
The membrane was cut into pieces by 5mm×5mm on which M13-DNA was placed with the designated concentration. The pieces were placed into Eppendorff-tubes separately and the enzyme buffer (glycine-NaOH, 0.1M, pH9.5) containing p-nitrophenyl phosphate (1mM), $MgCl_2$(1mM), and $ZnCl_2$(0.1mM) was added. The tubes were incubated at 37°C. The reactions were stopped by adding EDTA (50mM) in $K_2HPO_4$-KOH buffer(0.5M, pH10.3) and absorbances at 405nm of the supernatant were measured with Shimadzu Spectrophotometer (UV-265FD).
*Method(2) Colorimetric detection on membranes.*
The membrane (20mm×60mm) as described above was transferred to the enzyme-reaction buffer (Tris-HCl,0.1M, pH9.2) containing NBT (0.33mg/ml), BCIP (0.17mg/ml), $MgCl_2$ (50mM), and NaCl (0.1M) in a plastic bag and was incubated at 37°C(10). The appeared spots were examined by eyesight.
*Method(3) Fluorometic detection in solution.*
The membrane was cut as described above. The pieces were placed in the Eppendorff-tubes separately and the enzyme-reaction buffer (Glycine-NaOH, 0.1M, pH9.5) containing

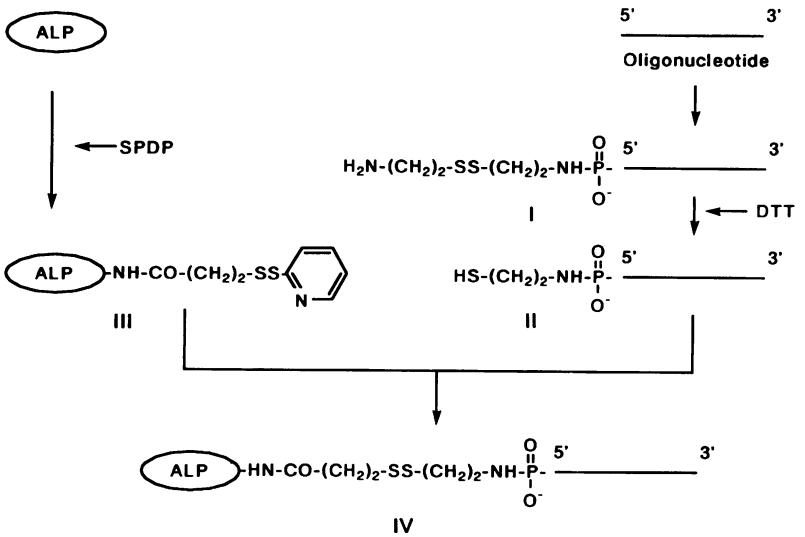
3'·· CAACATTTTGCTGCCGGTCACGGT··5'
5'GTAAAACGACGGCCAGT 3'
P17

**Fig.1** Partial sequence of M13mp8 phage DNA and its complementary oligonucleotide (P17).

MUP (0.01mM), $MgCl_2$(1mM), and $ZnCl_2$ (0.1mM) was added(11). The tubes were incubated at 37°C and the reactions were stopped by adding EDTA (50mM) in $K_2HPO_4$-KOH buffer (0.5M, pH10.3). Fluorometric intensities of the supernatants were measured by Shimadzu Fluorescent Spectrophotometer(RF-540) with $\lambda_{exc}$=360nm and $\lambda_{em}$=450nm.

## RESULTS AND DISCUSSION

The base sequence of oligonucleotide(P17) used was complementary to M13mp8 phage DNA as shown in Fig.1. The schematic route of the preparation of an alkaline phosphatase-linked oligonucleotide adduct was depicted in Scheme 1. The mediator between two species was used so that both oligonucleotide and enzyme can function independently. In this paper, combination of 2-aminoethanethiol moiety, which is derivatized from cystamine, and SPDP was adopted as the mediator. Cystamine first introduced to the 5'-phosphorylated oligonucleotide via a phosphoramidate linkage(I) was treated with DTT to generate to 2-thioethanol group(II). II reacted with SPDP-linked alkaline phosphatase(III) to give the enzyme-linked oligonucleotide conjugate(IV). These reactions were verified with HPLC, PAGE, and gel filtration chromatography. Figure 2 shows the HPLC and PAGE profiles of oligonucleotide modifications. P17(Fig.2(I)-(D)) was phosphorylated at 5' hydroxyl group with PNK. Thirty nanomoles of oligonucleotides were easily phosphorylated by using




**Scheme 1** Preparation of an alkaline phosphatase linked oligonucleotide as a DNA-probe.

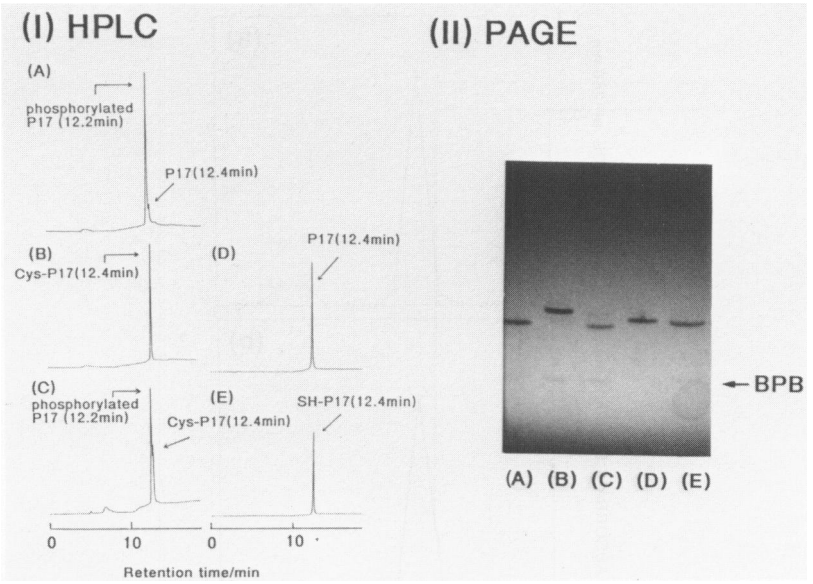



**Fig.2** HPLC(I) and PAGE(II) profiles of modified oligonucleotides.
(A) 5′-phosphorylated P17, (B) Cys-P17, (C) Cys-P17 after 80%AcOH treatment, (D) P17, (E) SH-P17.
Conditions of HPLC and PAGE were described in the EXPERIMENTAL.

30units of T4-PNK with a satisfactory yield as shown in Fig.2(I)-(A) and Fig.2(II)-(A). Introduction of cystamine to 5′-phosphate group was carried out according to Chu's method using water soluble carbodiimide(EDCI)(12,13). This reaction was almost quantitative (Fig2(I)-(B) and Fig 2(II)-(B)) and the product was isolated by HPLC. The product isolated was treated with acetic acid to verify the presence of an acid-labile phosphoramidate linkage and as shown in Fig.2(I)-(C) and Fig.2(II)-(C), the presence of the linkage was suggested. The cystamine linked P17(Cys-P17) was treated with DTT to cleave the bisulfide linkage of the cystamine of Cys-P17 and the reaction was monitored with PAGE. Appearance of the new band suggests the presence of Cys-P17 derivatives having a 5′-thiol group(SH-P17, Fig.2(I)-(E) and Fig.2(II)-(E)). After 4h, the reaction was almost completed and the product was isolated from HPLC. During evaporation or desalting of the fractions, it was found that the SH-P17 was converted to unknown side products. The reduction with DTT and the collection of the SH-P17 were carried out just before the cross-linking reaction with the enzyme.

Alkaline phosphatase from calf intestine(CAP) and from bacteria (BAP) were used. In the preliminary experiments, the heat stability and the detection limits of those enzymes were examined by colorimetric methods. It was found that BAP is more stable against heat treatment than CAP and that the detectable limit(moles) of BAP was much larger than CAP. This may be due to the large difference of their turnover numbers. The proposed turn over number of CAP ($5\times10^5$/min) is several hundred times larger than BAP. Here, CAP was utilized to attain the high sensitivity.

CAP was treated with SPDP, which is a hetero-bifunctional cross-linker and specific to both thiol and amino groups. As alkaline phosphatases have only a few numbers of

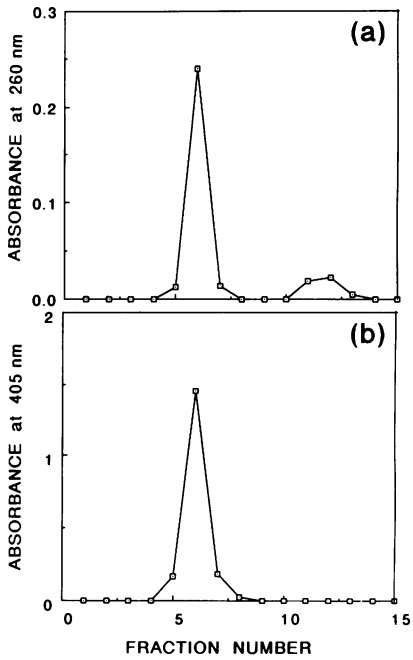



**Fig.3** Gel filtration chromatogram of the reaction solution of SH-P17 and SPDP-CAP.
(a) monitored at 260nm,
(b) Enzyme activities of the fractions monitored at 405nm after hydrolysis of p-nitrophenyl phosphate. Conditions were same as Method(1).

free thiol groups, it was expected that SPDP dominantly reacted with amino groups of CAP leaving a pyridylsulfide moiety intact. Absorbances due to 2-mercaptopyridine which was released by treatment SPDP-enzyme adduct with DTT, was monitored. It was found that one molecule of CAP carried nineteen molecules of SPDP. The SPDP-CAP was then treated with freshly prepared SH-P17. As shown in Fig.3 unreacted SH-P17 (Peak II) was separated from enzyme adduct(CAP-P17) by gel filtration chromatography. The molar ratio of P17 to CAP in CAP-P17 was calculated to be almost 1.8:1 using equation (1), A(260)/A(280)=1.16, and UV molar extinction coefficients at two different wavelengths. This means that one molecule of CAP carries almost two molecules of P17, which seems to be suitable for the use as a DNA-probe. CAP-P17 could be stored in PBS buffer (40mM) at 4°C for at least three months without any loss of its enzyme activity.

In order to estimate the ability of the CAP-P17 as a DNA-probe for detecting small amount DNA, a simple and convenient model system was set. A combination of M13mp8 phage DNA (single strand, M13-DNA) and its complementary primer was chosen as the model system. M13-DNA is popular DNA whose base sequence is known and is commercially available. Several sequences complementary to M13-DNA have been used as primers for DNA sequencing. Base sequence of P17(dGTAAAACGACGGCCAGT, Fig.1) is one of those primer sequences and is complementary to the downstream region of the cloning site of M13-DNA. This model system can be a universal control to evaluate

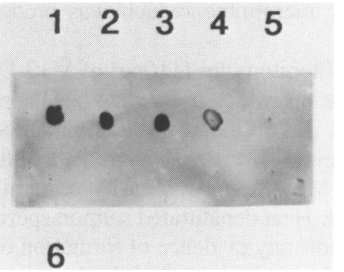



**Fig.4** Detection of M13-DNA by Method(2) using CAP-P17 and BCIP/NBT.
Amount of M13-DNA; 1. 50femtomol(110ng),2. 5femtomol (11ng), 3. 500attomol(1.1ng), 4. 50attomol(0.11ng), 5. 5attomol(0.011ng).
Amount of heat-denatured salmon sperm DNA; 6. 100ng.

the ability of DNA-probe systems. After hybridization, it is quite troublesome but unavoidable to separate unhybridized DNA probe from formed hybrid. While several trials have been reported to overcome this matter, they could not attain practical sensitivities. Hence, membrane had to be utilized to attain practical sensitivities; that is, detectable minimal amounts of target DNA range from 100attomole to 1attomol of target DNA. M13-DNA immobilized on nitrocellulose membrane was hybridized with enzyme-labeled DNA probe and M13-DNA and visualized with three methodology. They are colorimetric detection in solution (Method(1)), colorimetric one on membranes (Method(2)), and fluorometric one in solution (Method(3)).

Method(1) requires p-nitrophenyl phosphate as an substrate and p-nitrophenol released in the solution was monitored by UV spectrophotometer at 405nm. Method(2) requires 5-bromo-4-chloro-3-indolyl phosphate(BCIP) and nitroblue tetrazolium(NBT) and formed

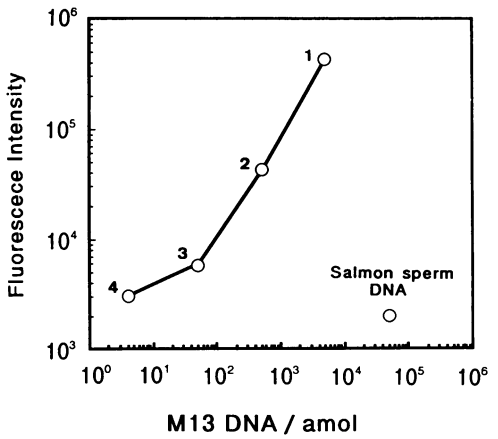



**Fig.5** Detection of M13-DNA by Method(3) using CAP-P17 and MUP.
Amount of M13-DNA; 1. 5femtomol(11ng),2. 500attomol (1.1ng), 3. 50attomol(0.11ng), 4. 5attomol(0.011ng).
Amount of heat-denatured salmon sperm DNA; 100ng.
The fluorescence intensities were obtained by subtracting those of control area where no DNA was fixed.

insoluble colored dye on the membrane, which was recognized by eyesight and by a chromatoscanner(10).

In the case of Method(1), 50femtomoles (110ng) of M13-DNA could be hardly detected even after several hours incubation, which means that this method is not suitable for practical uses unless target DNA/RNA is amplified by suitable methods such as PCR(13) and QB-RNA virus amplification system(14). Fig.4 showed the results of Method(2). After 60min incubation. 50 attomoles of M13-DNA was easily recognized by eyesight and even 5 attomoles could be recognized. Heat denaturated salmon sperm DNA (100ng) immobilized in the same manner did not show any evidence of formation of hybrid with CAP-P17. This result implies that non-specific binding of CAP-P17 to salmon DNA or the membrane was negligible in Method(2), even though the conditions of hybridization and washing were not very stringent. Those spots on the membrane were also analyzed by a chromatoscanner to obtain the calibration curve for the evaluation of amounts of target DNA and the trial was failed mainly because of diffraction of the monitoring light at the membrane surface and of saturated coloration when large amounts of DNA was detected (data not shown).

Fig.5 depicted the results of Method(3) after 30min incubation. The calibration curve was obtained by the least square calculation in the range from 5femtomoles (11ng) to 5attomoles (0.011ng) of target DNA. Heat-denaturated salmon sperm DNA (100ng) could not be recognized by this method, showing non-specific binding was also negligible in Method(3). As CAP-P17 showed high specificity in the recognition of DNA, the oligonucleotide in CAP-P17 seems to function as DNA-probe. Also, CAP could function with a satisfactory turnover numbers, even though amino groups were occupied by the cross-linker.

The efficiency of of enzyme reaction in the CAP-linked DNA-probe system was then estimated. CAP alone was placed on nitrocellulose membrane, air-dried, and visualized by Method(3). 0.5Attomoles of CAP was detected, whereas 5attomoles of CAP was the detectable limit in the CAP-P17 system. This difference in the sensitivity could be explained by three reasons as follows; (1) deactivation of the enzyme in the chemical modification steps, (2) deactivation of the enzyme during hybridization steps, and (3) low efficiency of the hybridization.

## CONCLUSION

Many methods to label DNA-probes have been reported. They were utilized for DNA detection in various conditions. Radio-isotopes may be the most popular and easiest method because DNA-probe has no special label which may cause the non-specific binding to membrane. Instead of radioisotopes, here enzyme was used as a label of DNA-probes. There are mainly three positions where labels are introduced. They are nucleobase moieties, phosphate groups in the internucleotide linkages, and 5′ or 3′ terminals. The phosphoramidate linkage, which was introduced by Orgel, was adopted in this report and was found to be stable during several steps of hybridization. A disulfide linkage located in the middle of the spacer was also found to be stable during hybridization. Thiol groups generated by DTT treatment of the cystamine moiety are readily attacked by thiol orienting functional groups such as maleimidyl and iodoacetyl groups. Any labels having such groups can be used for DNA-probe labelings.

In comparison of three methods examined here, both Method(2) and Method(3) showed satisfactory and characteristic results. For the application of DNA-probe methodology to

diagnostic uses, automation of routine procedures is quite essential. At least hundreds of samples must be handled and accurate diagnosis must be given within a few hours. Method (3) may be suitable for such purpose because the homogeneous solutions are easy to be detected by spectrophotometers in quantitative manners. On the other hand, records obtained from Method(2) remain as pictures like X-ray films after autoradiography.

Usage of enzyme-linked DNA-probes may be the best way so far, and automation of DNA-probe diagnosis is very probable. Depending on the samples or nature of the diagnosis, the method to detect target DNA's must be selected,i.e. Method (2), or Method (3). Both ways reported in this paper were found to be promising for the application to automatic diagnostic instruments.

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