Rapid Hybridization at High Salt Concentration and Detection of Bacterial DNA Using Fluorescence Polarization

Makoto Tsuruoka[1]* and Isao Karube[2]

[1]*Department of Electronics, School of Engineering, Tokyo University of Technology, Katakura 1404-1, Hachiouji, Tokyo, 192-0981 Japan*

[2]*Katayanagi Advanced Research Laboratories, Tokyo University of Technology, Tokyo, Japan*

Abstract: The effects of NaCl concentration and temperature on the rate of hybridization of complementary single-stranded DNA (24-mers) were investigated. The single label of fluorescein was used for the probe DNA. The time courses of fluorescence polarization for the probe DNA were monitored. It was shown that detection of a specific DNA sequence (24-mer) was possible in less than 10 min using fluorescence polarization under the optimized conditions of 0.8 M NaCl at 46°C in TE buffer. The effects of base-pair mismatches on DNA hybridization in the presence of NaCl or $MgCl_2$ were also investigated, and the specificity was considered by comparing the hybridization rate of the fluorescein-labeled probe. Determination of a specific DNA sequence was also possible in TE buffer containing 0.2 M $MgCl_2$. Moreover, in the presence of 0.2 M $MgCl_2$, there were no undesirable effects on hybridization and the presence of a single base pair mismatch could be identified. Rapid and specific determination of the DNA of enterohemorrhagic *Escherichia coli,* methicillin resistant *Staphylococcus aureus* and *Legionella pneumophila*, which had been multiplied by the asymmetric PCR, was performed under the optimized conditions for hybridization. It was confirmed that the conditions were also applicable to the hybridization between the probes and the amplified products of the actual bacterial genes. The combination of fluorescence polarization with the asymmetric PCR was quite effective. Moreover, the nested and asymmetric PCR product of bacterial gene could be detected effectively. The DNA detection method could also be used even if the specificity of the DNA amplification was not perfect and some unexpected bands were mixed with the target band during electrophoresis.

1. INTRODUCTION

Fluorescence polarization (FP) is a useful method in the research fields of immunochemistry and biochemistry. In immunochemistry, fluorescence polarization immunoassay (FPIA) is used as a rapid method for detecting antigens and haptens of relatively low molecular weight (less than ~ 10^4 Da) and does not require a process to separate bound and unbound assay components. In biochemistry, the interactions between bioactive molecules, for example proteins, have been investigated using FP.

In FP assays, membranes, micro-particles, gel or other immobilized sorbents are not needed, and the extent of hybridization can be measured as it occurs in solution without the need for separation steps. However, the optimization of reaction conditions and reagents are important in this method. In this article we describe optimization of the conditions for DNA hybridization and rapid detection of the microorganism genes. In order for an assay to have clinical or research utility for high throughput screening of DNA, the assay time should be as short as possible. In a previous report using FP, even though the immunoassay of protein was performed in less than 10 min [1], it took more than 10 h for the DNA hybridization or association to reach completion [2]. The time required for the measurement of DNA by FP could thus be considerably decreased if the rate of hybridization could be accelerated.

*Address correspondence to this author at the Department of Electronics, School of Engineering, Tokyo University of Technology, Katakura 1404-1, Hachiouji, Tokyo, 192-0981 Japan; Tel/Fax: +81-426-37-2405; E-mail: tsuruoka@cc.teu.ac.jp

DNA hybridization has been previously investigated using UV light absorbance [3,4], hydroxyapatite chromatography [5] and gel filtration [6]. Some methods based on energy transfer [7], the frequency shift of a quartz crystal device [8] and surface plasmon resonance [9] have also been proposed for evaluating DNA hybridization and detecting specific DNA sequences.

In the nucleic acid detection using FP, fluorescent-labeled oligo-DNA is usually used as a reporter molecule for obtaining the value of FP or anisotropy. DNA/RNA detection methods using FP can be classified into two main groups. In one method fluorescent-labeled oligo-nucleic acid is used as a primer, and the extension and/or amplification reaction of the nucleic acid is performed. The other method uses a fluorescent-labeled oligonucleotide as a probe, which is simply mixed with target samples. This method does not necessarily need combination with a gene amplification method. In this paper, the former is called the primer FP method and the latter is called the probe FP method. This article mainly addresses the probe FP method.

In the probe FP method, the optimization of the rate of DNA hybridization is particularly important for high throughput screening. It is a positive feature that the range of the hybridization conditions that can be chosen is very wide (Section 3.1). Moreover, because the probe DNA is simply mixed and hybridized to the target nucleic acid, the products of some different amplification methods and some nucleic acid samples without amplification can all be measured under the same reactive conditions in one series of experimental operations.

1386-2073/03 $41.00+.00

However, in the primer FP method or the FP integrated with an amplification method, the hybridization condition of the primer or primers is fundamentally restricted by the condition under which the reaction for DNA amplification or elongation can be performed. In section 3.1, optimization of the conditions for the rate of hybridization reaction between fluorescent-labeled oligo-DNA (probe DNA) and complementary oligo-DNA is reviewed. If the specificity of hybridization is low, even when the reaction rate is high, it is insufficient for the DNA detection by a detection system. Therefore in section 3.2, the specificity is investigated under the optimized conditions for hybridization.

Section 3.3 describes the methodology for the rapid detection of bacterial genes using the combination of FP and nucleic acid amplification. Since in the probe FP method, the product is measured after bacterial DNA amplification, high sensitivity at the same level as the gene amplification method can be achieved. In addition, the specificity can be confirmed at a level of probe hybridization. Pseudo-positive data can be avoided even in the case when the nucleic acid amplification is not very specific (due to the sample or reaction conditions). Many nucleic acid amplification techniques such as PCR, NASBA and SDA are now in practical use, and combinations of FP with those can be proposed. Here, we discuss whether the hybridization conditions between probe DNA and complementary oligo-DNA (examined and proposed in Sections 3.1 and 3.2), are applicable to the hybridization between a probe and an amplified product of bacterial gene. The amplification method mainly used is asymmetric PCR arranged for the probe FP method. The combination of the probe FP with NASBA is briefly mentioned in section 3.3. The combination with SDA is reviewed in another paper of this issue. In Section 3.3, the results of detecting the amplified products of actual bacterial genes with the probe FP method are also reviewed. Rapid hybridization has been achieved in less than one minute using the probe FP method [10]. Therefore, high throughput screening is possible using this method.

2. EXPERIMENTAL

2.1. Reagents and Materials

2.1.1. Materials

Phosphoramidite deoxyribonucleotide reagents (Perkin-Elmer, USA) and phosphoramidite fluorescein labelling reagent (FluorePrime, Pharmacia, Sweden) were used for synthesizing the oligonucleotides. TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA disodium salt) was used to dilute the solutions. Salmon sperm DNA (Funakoshi, Japan) was used as a negative control DNA sample. All chemicals were of the highest reagent grade.

2.1.2. Preparation of Target DNA, Probe DNA and Control DNA, and Hybridization Between the oligo-DNAs

Oligonucleotides were synthesized using a DNA synthesizer (392, Perkin-Elmer) and purified by HPLC (L-6200, Hitachi, Japan). The concentrations of the purified DNAs were determined from their absorbance at 260 nm.

The sequence of the target DNA T_0 corresponded to part of the gene coding the heat-labile enterotoxin of enterotoxigenic *Escherichia coli* [11]. The sequence of the probe DNA P was perfectly complementary to that of the target DNA T_0 and had a fluorescein label at its 5' terminus. A 30-base control oligomer C of random sequence, and three other target DNAs; T_1, T_3, and T_6, bearing the indicated number of mismatches against the probe DNA P were also synthesized.

The sequences of the oligomers are shown below. The underlined bases indicate the mismatches against the probe DNA P and F represents the fluorescein label.

T_0 5'- ATA CCG GTC TCT GTA TTC CCT GTT -3'

P 5'-F AAC AGG GAA TAC AGA GAC CGG TAT -3'

C 5'- CCA CCG GCA GTA GCG CAT CTA TGC ATT AGG -3'

T_1 5'- ATA CCG GTC TC<u>C</u> GTA TTC CCT GTT -3'

T_3 5'- ATA CC<u>A</u> GTC TC<u>C</u> GTA TTC <u>T</u>CT GTT -3'

T_6 5'- ATA <u>T</u>C<u>A</u> GT<u>T</u> TC<u>C</u> GTA <u>C</u>TC <u>T</u>CT GTT -3'

The DNA concentrations after mixing were 1 x 10^{-8} M for the probe DNA and 1 x 10^{-7} M for all the target and control DNAs. Measurements were performed immediately after adding the targets T_0, T_1, T_3, and T_6, or the control DNA C to the probe DNA P at 46°C, unless otherwise noted (Sections 3.1 and 3.2).

2.1.3. Reagents and Samples for Detecting the Verotoxin Gene of EHEC

The oligonucleotides were synthesized using the reagents (Sawady Technology, Japan). The probe DNA sequence, with a fluorescein label at its 5' terminus, was part of the gene encoding the Verotoxin type 2 (VT2) [12]. The oligonucleotide sequences of primer V2a, primer V2b [13], and the probe DNA are shown below.

V2a 5'- GAACGAAATA ATTTATATGT -3'

V2b 5'- TTTGATTGTT ACAGTCAT -3'

Probe DNA 5'- F AGTATCGGGG AGAGGATGGT GTC -3'

A feces sample identified as containing EHEC (enterohaemorrhagic *Escherichia coli*; O157) carrying VT2 gene by the Hiroshima City Meat-Inspection Office, was heated at 100°C for 10 min to extract DNA from the bacterium.

2.1.4. Reagents and Samples for Detecting the Gene of MRSA

The oligonucleotide sequences of primers (a), (b), (c), (d), and probe DNA (e) for detecting the DNA of MRSA (methicillin resistant *Staphylococcus aureus* [14]; are shown below.

(a) 5'- GAACCTCTGC TCAACAAGTT -3'

(b) 5'- TTACAACTTC ACCAGGTTCA -3'

(c) 5'- ACTGCCTAAT TCGAGTGCTA -3'

(d) 5'- TTCATGCCTT TTTCAAATTT -3'

(e) 5'- F ATCTTGTAAC GTTGTAACCA CCCCA -3'

The probe DNA sequence (e) was designed to be complementary to the DNA amplified by the primers (a), (b), (c) and (d). The primers (b) and (c) were used for

amplification of the nested sequence of the PCR product with the primers (a) and (d).

2.1.5. Reagents and Samples for Detecting the Gene of L. Pneumophila

Oligonucleotide primers and probe were designed from the partial sequences of a 16S-23S rRNA spacer region of *L. pneumophila* (*Legionella pneumophila*) in the Genebank database. The oligonucleotide sequences of primers Lp2F and Lp5R, and probe LpP2 are shown below.

Lp2F 5'- CATCCTCCTC GGCTCCACCA -3'

Lp5R 5'- CGCTCGTTTC CAGCTCCCC -3'

LpP2 5'- F GAAGAAGAGG TAACACAAGC GATTGG -3'

L. pneumophila serogroup 1- 6, *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. micdadei* were kindly contributed by Shiba (Jikei University School of Medicine), and *Pseudomonas aeruginosa* by Ohtake (Hiroshima University). These eleven isolates were incubated on α BCYE plates at 37°C for 3 days. DNA samples were then extracted from these colonies on the plates.

2.2. Amplification of the Genes of EHEC (Verotoxin), MRSA and *L. Pneumophila*

2.2.1. Verotoxin Gene Amplification

One μl of the heat-treated sample was subjected to standard DNA amplification using primers V2a, V2b (10 pmol each) and 2.5 units of DNA polymerase (Ex, Takara Shuzo, Ltd., Japan) [15]. The total volume of the reaction mixture was 100 μl. The PCR cycle was 30 sec at 94°C, 30 sec at 45°C and 1 min at 72°C. The sequence of 905 base pairs was amplified for 40 cycles. For the asymmetric PCR [16], 100 pmol of primer V2b was used and the other conditions were essentially the same with those for the standard PCR.

2.2.2. MRSA Gene Amplification

An MRSA sample isolated in a Japanese hospital for academic use was heat-treated and the sample was applied in standard PCR with the primers (a) and (d). The product was further applied in asymmetric PCR with the primers (b) and (c). For the asymmetric PCR, 100 pmol of one primer (ten times more than the other primer) was used.

2.2.3. L. Pneumophila Gene Amplification

Each template DNA (50 ng) was amplified by the asymmetric PCR. Front primer Lp2F (20 pmol) and reverse primer Lp5R (200 pmol) were used to amplify a 272 bp fragment of the 16S-23S rRNA spacer region of *L. pneumophila*. The PCR mixtures were initially denatured at 94°C for 2 min and then subjected to the three-temperature PCR cycle consisting of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec and extension at 72°C for 1 min. The last cycle was denaturation at 94°C for 30 sec and primer annealing at 60°C for 30 sec.

2.3. Detection of the Genes of EHEC (Verotoxin), MRSA and *L. Pneumophila*

Under the optimized conditions (0.8 M NaCl at 46°C in TE buffer; Section 3.1), the fluorescence polarization (*P* value) was measured every minute after adding 80 μl of either the amplified DNA sample or the control sample to 400 μl of the probe DNA. The final concentration of the probe was 1 nM. The time course of polarization of the probe DNA was measured for 25 min.

For detecting the gene of *L. pneumophila,* the final concentration of probe LpP2 was 0.5 nM and the polarization was measured at 42°C.

2.4. Apparatus

Polarization values were determined using a fluorescence spectrophotometer (FP-770 or FP-777, Japan Spectroscopic, Ltd.). The wavelength filters were diffraction gratings that were adjusted to a wavelength of 485 nm (with a bandwidth of 5 nm) for excitation and 525 nm (with a bandwidth of 10 or 20 nm) for emission. The sample was placed in a 10 x 10 or 2 x 7.5 [mm x mm] quartz cuvette in contact with a heating block equipped with a temperature controller. The FP value was measured once per minute.

DNA amplification was performed using a PCR thermal cycler (model 9600, Perkin-Elmer, U.S.A.). An automated electrophoresis system (PhastSystem, Pharmacia Biotech, Sweden) was used to confirm the DNA amplification (Section 3.3.1).

2.5. Application of FP Method

The equation describing FP is well known:

$$\frac{1}{P} = \frac{1}{P_0} + \left(\frac{1}{P_0} - \frac{1}{3} \right) \frac{RT\tau}{V\eta}$$

where the polarization P depends on the effective volume V of the fluorescent substance (molecular mass times molar volume), the coefficient of viscosity η of the solvent, the absolute temperature T, and the lifetime τ of the excited state of the fluorescent substance [17]. The equation will be explained in other chapters of this edition.). R is the gas constant and P_0 is the value of P at 0 K. Given that the temperature and viscosity of the solution are constant, it can be seen that polarization increases with the apparent molecular weight of a fluorescent-labeled substance. Thus DNA hybridization may be monitored if one strand of DNA is labeled with a fluorescent substance. This is a brief description of the probe FP method (Section 1).

2.6. Rate of DNA Hybridization

A simple model of DNA hybridization involves equilibrium between association and dissociation reactions as expressed below.

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB \qquad (1)$$

However, once the oligonucleotides are annealed, the rate of dissociation is negligibly small and the association process alone predominates, giving the equation below.

$$A + B \longrightarrow AB \qquad (2)$$

This considerably simplifies analysis of the reaction and yields the following equation:

$$d[A_t] / dt = - k [A_t] [B_t] \quad (3)$$

where k is the rate constant for hybridization and $[A_t]$ and $[B_t]$ are the concentrations of the reaction components at time t. In the case of $[B_0] >> [A_0]$, the following approximation is derived.

$$[AB_t] = [A_0] \{1 - \exp(- [B_0] k t)\} \quad (4)$$

where $[A_0]$ and $[B_0]$ represent the initial concentrations of A and B. Accordingly, we used the following equation to fit our data in the probe FP method:

$$\Delta P_t = w_1 \{1- \exp(- w_2 t)\} \quad (5)$$

where ΔP_t is the increase in FP at time t, w_1 is related to the initial concentration $[A_0]$ of the probe DNA, $w_2 = [B_0] k_h$ where $[B_0]$ corresponds to the initial concentration of the target DNA, and we define k_h as the rate constant for hybridization. In our experiments, the concentration of the target DNA was always 10-higher than that of the probe DNA.

Note, however that the relationship between the salt concentration and the rate of hybridization cannot be explained with this interpretation of the data. The assumption is given that the formulae hold in the range of the salt concentration and the reaction temperature. As can be readily seen in Figure **1**, the rate and extent of polarization is dependent upon the NaCl concentration. The hybridization does not go to completion but equilibrates with only a fraction ($f = [AB_\infty] / [A_0]$) of the available, fully annealed $[A_0]$ (and therefore the equivalent amount of $[B_0]$), where $[AB_\infty]$ represents the concentration of $[AB]$ at $t = \infty$. Thus both w_1 and w_2 contain a contribution due to f and reflect the influence of the NaCl concentration on the extent and rate of hybridization. For simplicity, w_1 may be considered as $f [A_0]$ and w_2 as $[B_0] k_h$. Thus the k_h obtained from mathematical fitting of the data is a constant for the relative rate of hybridization at a specific NaCl concentration.

Nevertheless, the data were almost perfectly fitted by equation (5) and, in most cases, the chi squared value [18] was on the order of 10^{-6} to 10^{-4}. Thus k_h could be calculated using the coefficients of the fitted curve so that the progress of the hybridization could be analyzed at least semi-quantitatively. In any case, fitting of the data to equation (5) is quite sufficient for determining graphically the best conditions for the hybridization.

3. RESULTS AND DISCUSSION

3.1. Optimization of the Rate of DNA Hybridization

3.1.1. Effect of NaCl Concentration on DNA Hybridization

The time courses of polarization to DNA hybridization at 36°C in the presence of different concentrations of NaCl are shown in Figure **1**. The probe DNA P, the target DNA T_0 and the control DNA C were dissolved in TE buffer containing various concentrations of NaCl respectively (Section 2.1.2). ΔP represents the difference between polarization of the probe DNA in the presence of the target DNA and the control DNA, which is practically the difference between the signal and the noise. As seen in Figure **1**, the degree and the rate of hybridization increased with an increase in NaCl concentration. A nonspecific reaction or binding of the probe with a random sequence (the control DNA) could not be observed over the same NaCl concentration range (data not shown).

As can be seen from the equation (Section 2.5), polarization is directly proportional to the effective volume of the fluorophore, the viscosity of the solution, and inversely proportional to the fluorescence lifetime. It is difficult, however, to separate the contributions of the changes in specific volume and fluorescence lifetime although the latter could be directly measured. Since no significant change in fluorescence intensity was observed upon mixing the probe with the control DNA, inter-molecular effects may be disregarded. Therefore, changes in polarization should be a result of DNA hybridization.

The dependence of the rate of hybridization on NaCl concentration is shown in Figure **2**. The hybridization rate was calculated from the functions fitted to the time course data partly shown in Figure **1**. In accordance with the standard protocol, time is represented in min. for this method. In Figure **2**, the hybridization rate in 0.2 M NaCl was 1 x 10^7 M^{-1} min^{-1}, which was ~10-fold higher than that in 0.01 M NaCl. Furthermore, the hybridization dependence on KCl concentration was almost the same as on NaCl concentration (data not shown).

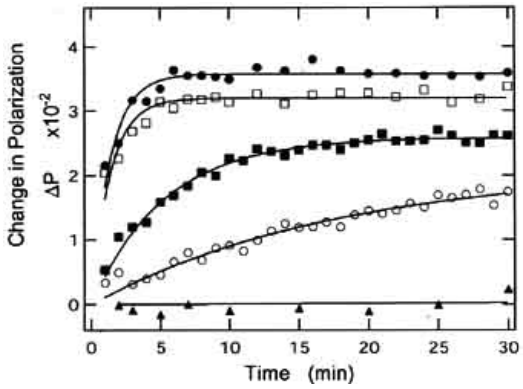

Fig. (1). Time course of the polarization to DNA hybridization in the presence of various concentrations of NaCl: (open circle), 0.01 M; (closed square), 0.04 M; (open square), 0.4 M; (closed circle), 3.2 M; (closed triangle), without NaCl; $[A_0] = 1.0 \times 10^{-8}$ M; $[B_0] = 1.0 \times 10^{-7}$ M; 10 mM TE buffer (pH 8.0), 36°C.

The maximum rate of 1.4 x 10^7 M^{-1} min^{-1} (2.3 x 10^5 M^{-1} s^{-1}) was achieved in the concentration range of 0.4 to 3.2 M. This rate is similar to the earlier reported rate of 3.6 x 10^5 M^{-1} s^{-1} obtained using size exclusion FPLC for an alkaline phosphatase-labelled 21 mer probe [6]. Although the rate of hybridization in the NaCl concentration range above ca. 1 M seemed to increase somewhat (Fig. **2**), NaCl would easily precipitate when storing the oligonucleotides at low temperatures. Given these considerations, the optimized NaCl concentration was determined to be 0.8 M.

3.1.2. Effect of Temperature on DNA Hybridization

The dependence of hybridization on the reaction temperature (26, 36, 46 and 56°C) was investigated. The concentrations of reagents after mixing were 1.8 x 10^{-9} M for the probe DNA and 1.8 x 10^{-8} M for the target DNA and the control DNA. The fitted curves were obtained using the equation (5), where the initial polarization values at 0 min for probe plus target DNA were taken to be the average polarization values of probe plus control DNA.

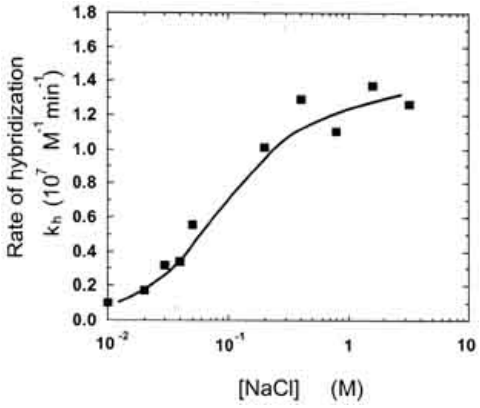

Fig. (2). Dependence of the rate of DNA hybridization on NaCl concentration: probe DNA, 1.0 x 10^{-8} M; target DNA and control DNA, 1.0 x 10^{-7} M; 10 mM TE buffer (pH 8.0), 36°C.

The hybridization was practically complete within 5 min at 46°C and 56°C. But the difference in polarization values between specific hybridization and nonspecific binding was larger at 46°C than at 56°C, indicating that the signal to noise ratio was greater at the lower temperature (data not shown; [19]. At 26°C and 36°C, the hybridization was not complete in 5 min. In view of the difference in polarization values between specific hybridization and nonspecific binding, a temperature of 46°C was used in all further experiments.

The time for DNA hybridization to reach 90% of completion was 3.7 min in 0.8 M NaCl at 46°C, as calculated from the fitting function. In the absence of NaCl at 46°C, the calculated (using approximately 40 min worth of data) time for 90% hybridization was ~ 70 h. This result was supported by previous data [2], where DNA hybridization was still not complete after more than 10 h. Under our optimized conditions (0.8 M NaCl at 46°C), DNA hybridization was greatly accelerated.

3.1.3. Calibration Curve for Target DNA

The calibration curve for the target DNA is shown in Figure **3**. The final concentration of the probe DNA P after mixing the target DNA T_0 or control DNA C with the probe DNA was 1.0 x 10^{-9} M. FP measurement was performed 10 min after adding various concentrations of target DNA or control DNA to the probe DNA under the optimized conditions (0.8 M NaCl, 46°C) in order to ensure complete hybridization. Each plotted value was the average of two measurements.

It should be noted that the optimal hybridization conditions are different for different assay methods [5-7]. From Figure **3**, the assay range was 10^{-10} M - 10^{-8} M, with a lower detection limit of approximately 10^{-10} M (300 fmol/assay). The sample volume could be reduced to one-seventh using a mini-cell so that the limit was approximately 40 fmol/assay.

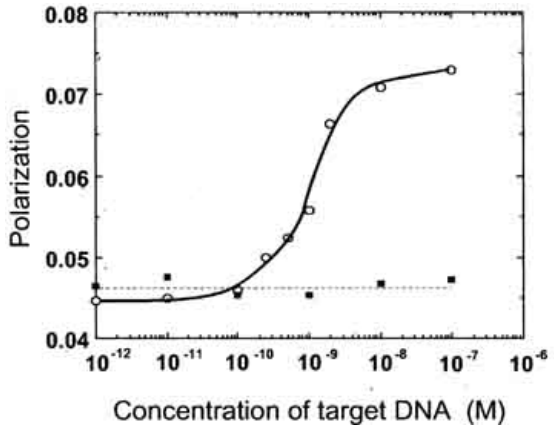

Fig. (3). Calibration curve for target DNA: (open circle), target DNA; (closed square), control DNA; the concentration of the probe DNA, 1.0 x 10^{-9} M; 10 mM TE buffer (pH 8.0), 0.8 M NaCl, 46°C.

3.2. Specificity: Detection of Complementary- and Mismatched DNA Sequences

3.2.1. Effect of Base-Pair Mismatches on DNA Hybridization in the Presence of NaCl

The effect of base-pair mismatches on DNA hybridization under the optimized conditions (0.8 M NaCl at 46°C) is

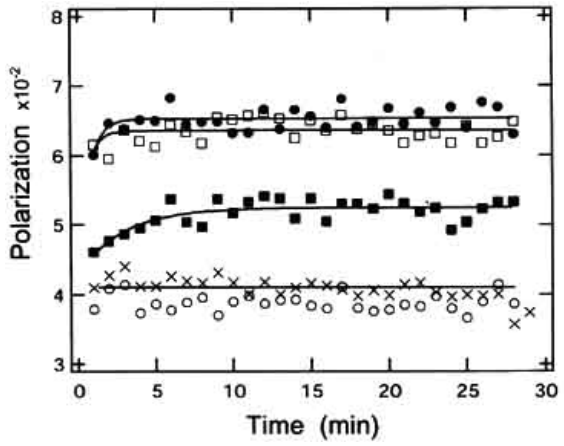

Fig. (4). Effect of mismatches on DNA hybridization in the presence of 0.8 M NaCl: target DNA with (closed circle) no mismatch, (open square) 1 mismatch, (closed square) 3 mismatches, (open circle) 6 mismatches and (x) control DNA; [probe oligomer], 1.0 x 10^{-8} M; [target and control oligomers], 1.0 x 10^{-7} M; 10 mM TE buffer (pH 8.0), 46°C.

shown in Figure **4**. The more mismatches introduced into the target DNAs, the slower was the hybridization. Average polarization values over 10 min (Av_{10}) were calculated for the statistical certification test. The differences between T_0, T_3 and T_6 were assured at confidence levels of greater than 99.9% while that between T_0 and T_1 (no mismatches and one mismatch) at a confidence level of less than 99.9%. There was no difference between the Av_{10} values of the control DNA C and the six-mismatch target DNA T_6.

Based on these results, the detection of target DNA should be possible (within 10 min) if a DNA sample had no or 1 mismatch against a labeled probe DNA. However, it was difficult to distinguish the mismatch of one base against the sequence P under the condition of 0.8 M NaCl.

3.2.2. Effect of Base-Pair Mismatches on DNA Hybridization in the Presence of $MgCl_2$

It has previously been noted that divalent cations such as Mg^{2+} can alter the structure of nucleic acid oligomers by coordination [20] and thus an effect on hybridization rate might be expected. Indeed, in the presence of divalent cations the effect of base-pair mismatches can even be amplified. Thus the effect of $MgCl_2$ on the hybridization of complementary and mismatched DNA sequences was investigated.

The effect of base-pair mismatches on DNA hybridization in TE buffer containing 0.2 M $MgCl_2$ is shown in Figure **5**. These results are similar to those in Figure **4**. The hybridization was slower when more mismatches were introduced into the target DNA. In this case, however, the determination of one mismatch was possible at confidence levels of more than 99.9%. This presents the possibility of the determination of even a single mismatch in complementary DNA oligomers within 10 min. These results are quite important for the application of this method to specific DNA determination because magnesium ions could easily contaminate the analyte sample containing the cultured microorganism or PCR-multiplied DNA sample. Our data showed no undesirable effects in their presence (at least up to 0.2 M).

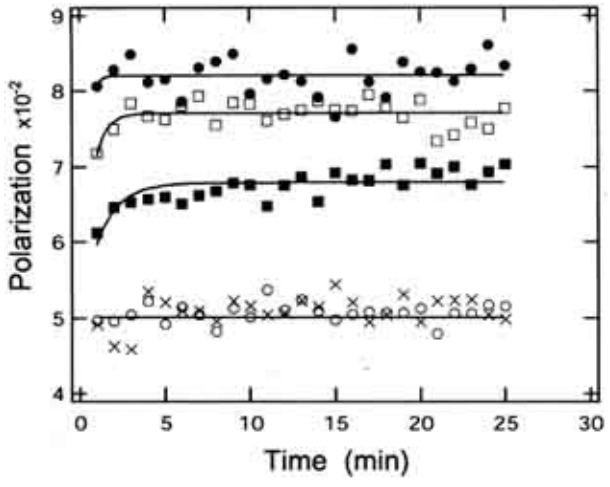

Fig. (5). Effect of mismatch on time course of DNA hybridization in the presence of 0.2 M $MgCl_2$: target DNA with (closed circle) no mismatch, (open square) 1 mismatch, (closed square) 3 mismatches, (open circle) 6 mismatches, and (x) control DNA; [probe oligomer], 1.0 x 10^{-8} M; [target and control oligomers], 1.0 x 10^{-7} M; 10 mM TE buffer (pH 8.0), 46°C.

3.2.3. Relationship Between Base-Pair Mismatches and the Rate of Hybridization

The relationship between the number of mismatches introduced into the target DNA and the hybridization rate in the presence of NaCl or $MgCl_2$ is shown in Figure. **6**. In the presence of 0.8 M NaCl or 0.2 M $MgCl_2$, the more mismatches introduced into the target DNAs, the lower was the hybridization rate and the weaker was the affinity between the target- and the probe DNA. In the presence of either salt, the hybridization rate for the target DNA T_6 containing 6 mismatches was nearly zero. It should be noted that the maximum rate observed in the presence of 0.8 M NaCl was 1.5 x 10^7 M^{-1} min^{-1} (2.5 x 10^5 M^{-1} sec^{-1}).

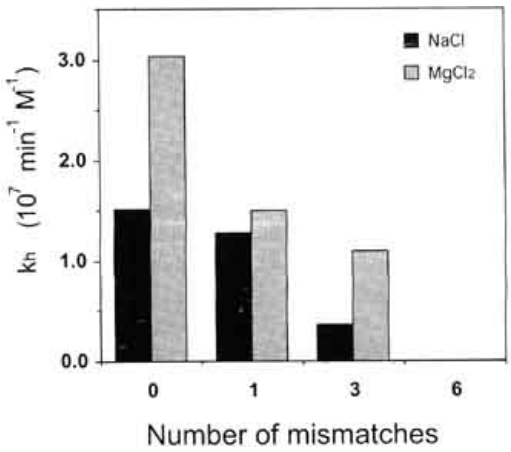

Fig. (6). Dependence of hybridization rate k_h on base-pair mismatches in the presence of 0.8 M NaCl or 0.2 M $MgCl_2$: [probe oligomer], 1.0 x 10^{-8} M; [target and control oligomers], 1.0 x 10^{-7} M; 10 mM TE buffer (pH 8.0), 46°C.

In the presence of 0.2 M $MgCl_2$, the detection of even a single base-pair mismatch was possible by comparing the calculated hybridization rates or, even more simply, the Av_{10} values of a target with those of a perfectly complementary oligomer. In the presence of 0.8 M NaCl, however, this was not possible.

3.3. Combination: Amplification of Bacterial DNA and Detection Using FP

Although the use of DNA amplification should facilitate highly sensitive detection of bacterial genes, initial attempts to use FP to detect bacterial genes amplified using standard PCR did not show large or reproducible changes in polarization. When using the probe oligo-DNA in association with FP, the probe would not hybridize with the target double-stranded DNA of longer chain length, because the target ds-DNA were more stable than the hybrid of the probe and the target ss-DNA. Therefore, the combination of

the probe FP method (Section 1) with asymmetric PCR was proposed. In the asymmetric PCR, the single-stranded DNA with which the probe DNA could hybridize was amplified much more than the other ss-DNA, which would compete with the probe DNA in the course of hybridization. So it was necessary to confirm the prediction and reveal the utility of the asymmetric DNA amplification for DNA detection using the probe FP method.

It was noted that other gene amplification methods, especially those in which ss-DNA or RNA would be amplified, could also be applied to the probe FP method. For instance, isothermal nucleic acid sequence based amplification (NASBA) is known for the amplification of (single-stranded) RNA with template RNA. It could be also used for the FP [21].

3.3.1. Rapid Detection of Escherichia Coli *Verotoxin Gene*

EHEC (enterohaemorrhagic *Escherichia coli*) can produce two types of verotoxin, VT1 or VT2. The former is the same as the toxin produced by *Shigella dysenteriae,* with no or single amino acid substitution. The latter (VT2) is considered original in EHEC and is suspected to be more harmful than VT1 and cause hemolytic uremic syndrome (HUS) in humans. Verotoxin types 1 and 2 (VT1 & VT2) are also called Shigatoxin types 1 and 2 (Stx1 & Stx2). In this section, the rapid detection of the gene using the probe FP method is described in conjunction with asymmetric PCR.

Time Course of Probe DNA Polarization

Time courses of the polarization of the probe DNA immediately after being mixed with the samples are shown in Figure **7** [22]. The samples were EHEC positive amplified using asymmetric PCR (Asym), EHEC positive using standard PCR (Sym), salmon sperm DNA using asymmetric PCR (C1), and EHEC positive without PCR (C2). As seen in Figure **7**, the time courses of the EHEC positive samples (Asym and Sym) were obviously different from those of the control samples (C1 and C2). Thus, the distinction between positive and negative samples was possible within 10 min of mixing the probe DNA with the samples. When the unamplified EHEC positive sample (1 μl) was added to the probe DNA, the polarization was as low as that of the salmon sample (negative control). This shows that the DNA amplification was both effective and necessary for the detection process.

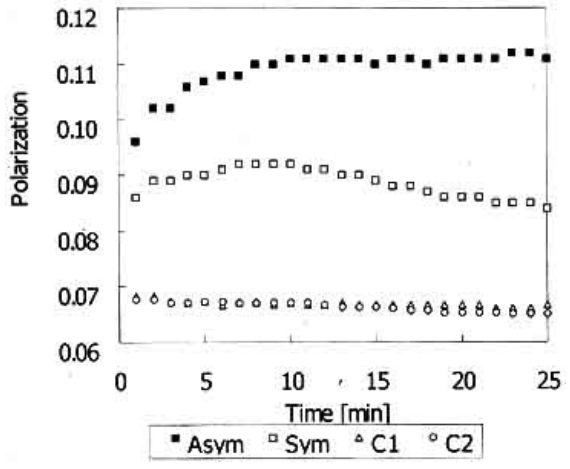

Fig. (7). Time courses of polarization of the probe DNA.

Samples: (Asym), an EHEC positive sample amplified using asymmetric PCR; (Sym), an EHEC positive sample using standard PCR; (C1), Salmon sperm DNA using asymmetric PCR; (C2), an EHEC positive sample without PCR.

Determination of the Verotoxin Gene

The polarization of the probe DNA after being mixed with the samples is shown in Figure **8**. Each bar represents the mean of the *P* value at 8, 9 and 10 min after mixing. The *P* values of the EHEC positive samples using both standard and asymmetric PCR were higher than those of the control samples (Sal, C1, C2). Moreover, the *P* values of the asymmetrically amplified PCR samples were sufficiently greater than those of standard PCR.

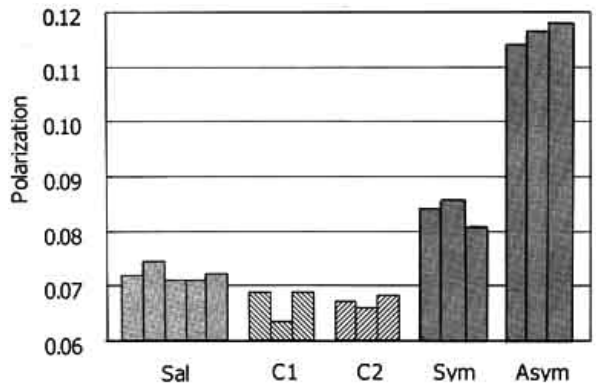

Fig. (8). Polarization of the probe DNA 10 min after mixing the probe with the samples.

Samples: from left to right, (Sal), Salmon sperm DNA (64 ng each); (C1), Salmon sperm DNA amplified using asymmetric PCR; (C2), an unamplified EHEC positive sample (1 μl each); (Sym), an EHEC positive sample using standard PCR; (Asym), an EHEC positive sample using asymmetric PCR.

In the asymmetric PCR, the concentrations of the primers were 10 to 1, so that the single-stranded DNA with which the probe DNA could hybridize would be amplified more than the other ss-DNA. The results supported our predictions and revealed the utility of asymmetric DNA amplification for DNA detection using fluorescence polarization (Section 3.3). When the salmon sperm DNA (80 ng) was used in the asymmetric amplification process, the polarization values were quite low (Fig. **8**, C1). This shows the high specificity of the method. Several EHEC negative samples were also used in the amplification, and the results were the same as those using the salmon DNA (data not shown).

When salmon DNA (Fig. **8**, Sal, 64 ng each) was added directly to the probe DNA, the *P* values seemed a little higher than those of the control samples (C1, C2). This may have been caused by some DNA binding proteins contained in the sperm DNA sample. It should be noted that, in this experiment, the minimum *P* values were in the range from

0.06 to 0.07, because the apparent volume of the probe DNA increases upon hybridization to the target DNA, and the *P* value increases with increasing apparent molecular volume.

Electrophoresis of the Samples Amplified Using PCR

In order to confirm the amplification of the target DNA, the PCR products that were measured using FP were analyzed using electrophoresis [22]. In both the standard and asymmetric PCR products of an EHEC positive samples, bands of *ca.* 900 bases in length were observed. Therefore the target DNA of VT2 must have been amplified. In the products of an EHEC negative sample, no bands of this size could be seen. The polarization of positive and negative samples showed excellent agreement with the results obtained from electrophoresis. However, no difference could be observed between the bands of samples amplified using standard and asymmetric PCR. Thus the amplification of the single-stranded DNA could not be confirmed using electrophoresis, while the advantageous effects of asymmetric PCR could be readily noticed using fluorescence polarization.

3.3.2. Rapid Detection of Methicillin Resistant Staphylococcus Aureus *DNA*

Rapid and specific determination of the DNA of methicillin resistant *Staphylococcus aureus* (MRSA), which had been multiplied by the nested and asymmetric PCR, was performed under the optimized condition of high salt concentration [23]. The sequence of the probe DNA in the probe FP method was complementary to a part of the gene encoding the structural protein responsible for the resistance of MRSA to methicillin [14]. Time courses of the polarization of the probe DNA (e) immediately after mixing with the samples are shown in Figure. **9**. The samples were MRSA positive, amplified using the nested and asymmetric PCR and the negative control (64 ng DNA of salmon sperm).

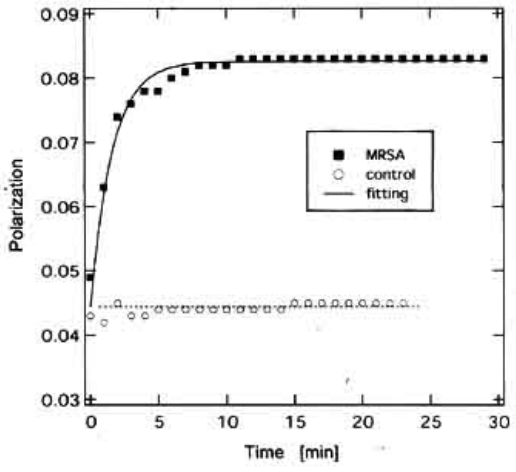

Fig. (9). Time courses of polarization of the probe DNA with the samples.

Samples: (MRSA), an MRSA positive sample amplified using the nested and asymmetric PCR; (control), the salmon DNA.

The curve in Figure **9** was fitted to the data using the approximate equation for hybridization (Section 2.6). The profile of the time course of MRSA sample was in good agreement with the results using synthesized model target DNAs (Sections 3.1 and 3.2). The difference of polarization between the positive sample and the negative control was more than the difference between the complementary oligo-DNA 25-mers and the same negative. This result was quite imporant, considering the difference between the molecular weights of the amplified DNA and the oligo-DNA. The time course of the MRSA positive sample was obviously different from that of the control negative sample. Thus, the distinction between positive and negative samples was possible within 10 min of mixing the probe DNA with the samples.

This method is capable of determining sensitively sequence specific DNA, because once amplified by PCR, the sequence of the DNA may be further confirmed by DNA hybridization. In this case, it would be much more specific because the nested PCR sample could be determined by the probe FP method.

3.3.3. Rapid Detection of the Gene of Legionella Pneumophila

Legionella pneumophila is a gram-negative aerobic, facultative intracellular bacterium and the causative agent of both Legionnaires' disease and Pontiac fever [24]. The sequences of PCR primers and probe for FP detection of this gram negative bacterium were in the intergenic 16S-23S ribosomal spacer regions, which have been used for identification of various genera at the species level [25]. The bands were detected near 272 bps for all PCR products except *L. micdadei* and *P. aeruginosa* using electrophoresis (Figure **10**). Therefore, *L. pneumophila* could not be distinguished from the other *Legionella* spp. by agarose gel electrophoresis.

Fig. (10). Agarose gel (1.2%) electrophoretic patterns of the asymmetric PCR products from various *Legionella* spp. and *P. aeruginosa* DNAs. Lanes 1- 6, *L. pneumophila* serogroup 1- 6; Lane 7, *L. bozemanii*; Lane 8, *L. dumoffii*; Lane 9, *L. gormanii*; Lane 10, *L. micdadei*; Lane 11, *P. aeruginosa*; Lane 12, the negative control.

Figure **11** shows FP measurements 5 min after mixing the probe with the samples [26]. Each polarization was the average of the three values. A few minutes after mixing the probe DNA, only the polarization for *L. pneumophila* isolates increased. Thus FP can distinguish *L. pneumophila* isolates from the others within five minutes.

Additionally, the minimum detectable value of *L. pneumophila* was investigated by using serial dilution of bacterial suspensions. There was obvious difference between

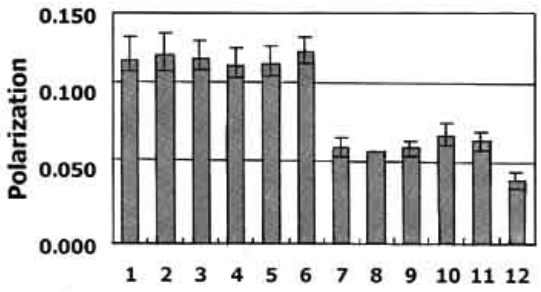

Fig. (11). Polarization of asymmetric PCR products from various *Legionella* spp. and *P. aeruginosa* DNAs, five minutes after mixing the probe. 1- 6, *L. pneumophila* serogroup 1- 6; Lane 7, *L. bozemanii*; Lane 8, *L. dumoffii*; Lane 9, *L. gormanii*; Lane 10, *L. micdadei*; Lane 11, *P. aeruginosa*; Lane 12, the salmon sperm DNA.

polarization for 10^3, 10^4 or 10^5 cells of bacteria and that for the control. Although the response for 10^2 cells was on average slightly higher than the control, there was no significant difference. Therefore, the minimum detectable value was 10^3 cells.

The standard method for detecting *Legionella* is culture, but the time required for culturing and confirmation requires several days [24]. The detection of amplified products using Southern hybridization is sensitive, but the process is tedious and requires considerable expertise. However, the PCR-FP method can easily and rapidly detect *L. pneumophila*. In this probe FP-PCR method, the minimum detectable count was 10^3 cells. In a standard, the minimal detectable level of *L. pneumophila* in water is more than 10^3 cells/L [27]. Therefore an improvement in the limit detection is very much needed. We have been working in this direction and recently, the detection limit has been improved to the level of 10^0 or 10^1 cells of bacteria by the technical improvement of DNA extraction from the microorganism sample (data not shown).

4. CONCLUSIONS

4.1. Fundamentals

The effects of NaCl concentration and temperature on the rate of hybridization of complementary single-stranded DNA 24-mers were investigated. It was shown that detection of a specific DNA sequence 24-mers was possible in less than 10 min using FP assay under the optimized conditions of 0.8 M NaCl at 46°C in TE buffer. Furthermore, this method was useful for evaluating the rate of DNA hybridization.

The effects of base-pair mismatches on DNA hybridization in the presence of NaCl or $MgCl_2$ were also investigated. It was shown that determination of a specific DNA sequence was also possible in less than 10 min at 46°C in TE buffer containing 0.2 M $MgCl_2$. Moreover, in the presence of 0.2 M $MgCl_2$, there were no undesirable effects on hybridization and the presence of a single base pair mismatch could be identified. These results are important for the application of this method to the rapid and specific determination of DNA sequences.

4.2. Possibility for High Throughput Screening of Nucleic Acids

The optimized conditions for hybridization between probe oligo-DNA and complementary oligo-DNA were also applicable to the hybridization between a probe and an amplified product of an actual bacterial gene. The use of 0.8 M NaCl was also appropriate for other DNA detection methods, since the hybridization conditions were not restricted by those for DNA amplification in the probe FP method.

To achieve highly sensitive detection of a microorganism, the FP method was used in conjunction with gene amplification and, furthermore, the combination with asymmetric PCR was found to be quite effective. Moreover, the nested and asymmetric PCR product of bacterial gene or the PCR product from the gene of 16S-23S ribosomal spacer region could be effectively detected using the probe FP method. The DNA detecting method could be used even if the specificity of the DNA amplification was not perfect and some unexpected bands were mixed with the target band, because the method is based on probe DNA hybridization.

The method could also be interesting as a basic research toll [2], since the DNA hybridization reaction can be followed by observing the time course of FP [19,28]. For example physico-chemical analysis of nucleic acid hybridization in the field of antisense drugs, which is usually followed using the UV melting method [29], may now be carried out using FP.

Rapid hybridization in less than one minute has been demonstrated using the probe FP method, and a variety of hybridization rates have been observed between probe DNAs and PCR products of bacterial genes [10]. Therefore, a combination of high rate probes for hybridization and high throughput detection or screening of nucleic acids using FP may be proposed. Furthermore, a very simple and single label of fluorescein may be used for the probe DNA, which will reduce reagent costs in this method. In conclusion, this method might be effective for the large-scale screening of DNA.

ABBREVIATIONS

Av_{10}	=	The average values of polarization over 10 min
BCYE	=	Buffered charcoal yeast extract
EHEC	=	Enterohemorrhagic *Escherichia coli*
FP	=	Fluorescence polarization
k_h	=	The rate constant for hybridization
L. pneumophila	=	*Legionella pneumophila*
MRSA	=	Methicillin resistant *Staphylococcus aureus*
NASBA	=	Nucleic acid sequence based amplification
PCR	=	Polymerase chain reaction
P value	=	Polarization value

rRNA	=	Ribosomal RNA
SDA	=	Strand displacement amplification
ss-DNA	=	Single-stranded DNA
TE	=	10mM Tris-HCl/1mM EDTA
UV	=	Ultraviolet
VT1	=	Verotoxin type 1
VT2	=	Verotoxin type 2

REFERENCES

[1] Tsuruoka, M., Tamiya, E., Karube, I. *Biosensors & Bioelectronics* **1991,** *6*, 501-505.

[2] Herning, T., Kobayashi, S., Tamiya, E., Karube, I. *Anal. Chim. Acta* **1991,** *244*, 207-213.

[3] Marmur, J., Doty, P. *J. Mol. Biol.* **1961**, *3*, 585-594.

[4] Wetmur, J. G., Davidson, N. J. *Mol. Biol.* **1968,** *31*, 349-370.

[5] Ness, J. V., Hahn, W. E. *Nucleic Acids Res.* **1982**, *10*, 8061-8077.

[6] Podell, S., Maske, W., Ibanez, E., Jablonski, E. *Mol. Cell Probes* **1991**, *5*, 117-124.

[7] Morrison, L. E., Stols, L. M. *Biochemistry* **1993**, *32*. 3095-3104.

[8] Okahata, Y., Matsunobu, Y., Ijiro, K., Mukae, M., Murakami, A., Makino, K. *J. Am. Chem. Soc.* **1992**, *114*, 8299-8300.

[9] Wood, S. J. *Microchemical journal* **1993**, *47*, 330-337.

[10] Tsuruoka, M., Murano, S., Okada, M., Ohiso, I., Fujii, T. *Biosensors & Bioelectronics* **2001**, *16*, 695-699.

[11] Tamatsukuri, S., Yamamoto, K., Shibata, S., Leno, F., Honda, T., Miwatani, T. *Eur. J. Clin. Microbiol. Infect. Dis.* **1991**, *10*, 1048-1055.

[12] Jackson, M. P., Neill, R. J., O'Brien, A. D., Holmes, R. K., Newland, J. W. *FEMS Microbiol. Lett.* **1987**, *44*, 109-114.

[13] Lin, Z., Kurazono, H., Yamasaki, S., Takeda, Y. *Microbiol. Immunol.* **1993**, *37*, 543-548.

[14] Matsuhashi, M., Song, M. D., Ishino, F., Wachi, M., Doi, M., Inoue, M., Ubukata, K., Yamashita, N., Konno, M. *J. Bacteriology* **1986**, *167*, 975-980.

[15] Erlich, H. A., Gelfand, D. H., Saiki, R. K. *Nature* **1988**, *331*, 461-462.

[16] Gyllensten, U. B., Erlich, H. A. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 7652-7656.

[17] Weber, G. *Adv. Prot. Chem.* **1953**, *8*, 415-459.

[18] Press, W. H., Teukolsky, S. A., Vetterling, W. T., Flannery, B. P. **1988**, Numerical Recipes in C. Cambridge University Press.

[19] Tsuruoka, M., Yano, K., Ikebukuro, K., Nakayama, H., Masuda, Y., Karube, I. *J. Biotechnol.* **1996**, *48*, 201- 208.

[20] Saenger, W. **1984**, Principles of nucleic acid structure. Springer Verlag. New York.

[21] Tsuruoka, M., Fujii, T. Nucleic Acids Symposium Series Oxford University Press **1999**, *42*, 239- 240.

[22] Tsuruoka, M., Fukuhara, K., Murano, S., Okada, M., Honda, T., Karube, I. *Trends Anal. Life Sci.* **1997**, *1*, 131-135. Electronic publication available at http://neo.pharm.hiroshima-u.ac.jp/tals/

[23] Tsuruoka, M., Fukuhara, K., Murano, S., Okada, M. *Nucleic Acids Symposium Series* **1998**, *39*, 115-116.

[24] Mahbubani, M. H., Bej, A. K., Miller, R., Haff, L., Di-Cesare, J. L., Atlas, R. M. *Mol. Cell Probes* **1990**, *4*, 175-187.

[25] Saruta, K., Matsunaga, T., Hoshina, S., Kono, M., Kitahara, S., Kanemoto, S., Sakai, O., Machida, K. *FEMS Microbiol. Let.* **1995**, *132*, 165-170.

[26] Fujii, T., Ohta, M., Kono, M., Hoshina, S., Fukuhara, K., Tsuruoka, M. *Nucleic Acids Symposium Series* **1999**, *42*, 59-60.

[27] Japan Minist. Health, Labour and Welfare, Environ. Health Bur., Planning Div. ed. **1994**, Guideline for the control of Legionellosis. Building management education center, p.p. 19-20.

[28] Tsuruoka, M., Yano, K., Ikebukuro, K. McNiven, S., Takeuchi, T., Karube, I. *Anal. Lett.* **1996**, *29*, 1741- 1749.

[29] Sugimoto, N., Katoh, M., Nakano, S., Ohmichi, T., Sasaki, M. *FEBS Lett.* **1994**, *354*, 74-78.